Food Research

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SUITABILITY OF 2,4-DINITROPHENYLHYDRAZINE METHODS FOR DETERMINING DEHYDRO- AND TOTAL ASCORBIC ACID IN RAW AND COOKED VEGETABLES

I. DEHYDROASCORBIC ACID[a]

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(Manuscript received April 29, 1958)

The 2,4-dinitrophenylhydrazine (DNPH) method for determining ascorbic acid of Roe and co-workers *(7, 8, 9)* has generally yielded results which agree well with those found by indophenol methods. However, studies of the homogeneity of DNPH derivatives prepared from natural products *(6)* as well as analyses of foods subjected to severe heat treatments *(2)* have indicated the procedures may lack the desired specificity.

In the present study, DNPH derivatives prepared under the usual analytical conditions for determining total ascorbic acid (AA) and dehydroascorbic acid (DHAA)[b] in vegetable extracts were purified by chromatographic procedures. Concentrations of AA determined from the fraction most closely resembling a reference compound prepared from crystalline AA were compared with those found by the usual DNPH methods. Studies of DHAA in unoxidized vegetable extracts are presented in Part I; those of total AA in oxidized extracts in Part II.

Determination of DHAA presents some problems different from the ones associated with the determination of total AA. Generally, DHAA constitutes a relatively small part of the total AA content of fresh vegetables *(3, 4, 5)*. Thus, if non-AA components are present the ratio of the concentration of these components to that of DHAA would be greater than that of the components to total AA. In addition, the possibility of interference by reduced AA exists in unoxidized extracts.

METHOD

Preparation of Vegetable Samples. Cooking methods were those described by Halliday and Noble *(1)* for cooking in boiling water. Cabbage samples were blended with 8 parts by weight of 6% metaphosphoric acid; asparagus with 4 parts. Blended samples were centrifuged and filtered. Cooking waters were diluted with an equal volume of 12% metaphosphoric acid.

2,4-Dinitrophenylhydrazine (DNPH) Method. Procedures of Roe and co-workers *(7, 8, 9)* were combined into a general procedure referred to here as the DNPH method. The DNPH reagent consisted of a 1% solution of DNPH in 4.5 M. sulfuric acid containing 1% thiourea. An incubation period of 3 hours at 37.0 ± 0.5° C. was used. Following incubation, 12.5 M. sulfuric acid was employed to dissolve the DNPH derivative and the percent transmission of this solution at 520 mμ was determined.

Chromatographic analysis of DNPH derivatives. DNPH derivatives for chromatographic analysis were prepared under the same conditions used for the DNPH method except that the amount of each component in the reaction mixture was increased 100 times. That the difference in amounts did not affect the reaction was shown by approximately equal yields of DNPH derivative being obtained (0.189 and 0.184 mg. per ml. of original solution) regardless of whether small or large amounts of reactants were used.

[a] Scientific Journal Series Paper No. 3932, Minnesota Agricultural Experiment Station.

[b] According to Roe, Mills, Oesterling, and Damron *(8)* determinations of DHAA in unoxidized extracts represent a composite of DHAA and diketogulonic acid.

At the end of the incubation period, the reaction mixture was cooled and filtered. The residue was dried thoroughly and then extracted with acetone. Aliquots were used for determining the absorption spectrum and for purification on chromatographic columns. In the former, acetone was removed and the residue dissolved in 12.5 M. sulfuric acid. The optical density was then read over the range of 330 to 600 mμ with a Beckman model DU spectrophotometer. In the latter, analysis was based on a procedure of Probst and Schultze *(6)*. Adsorbent was a mixture of 3 parts by weight of magnesium phosphate and one of Hyflo Super-Cel. The chromatogram was developed with acetone. It was cut at the boundaries of the fractions which remained on the column. These fractions were eluted with acetone containing 0.1 M. oxalic acid, the acetone evaporated, each residue dissolved in 12.5 M. sulfuric acid, and the optical densities determined.

Recoveries approximating 100% proved that losses did not occur during chromatographing. Thus, for 5 derivatives prepared independently from AA (U.S.P. crystals), the mean recoveries [c] were 98 at 530 mμ, 100 at 450 mμ, and 101 at 370 mμ. Furthermore, those for derivatives prepared from raw and cooked vegetables and cooking waters lay between 99 and 102.

Calculation of the concentration of DNPH derivative in its sulfuric acid solution was based on the optical density of the solution at 520 mμ and the extinction coefficient ($E^{1\%}_{1\ cm.}$) of a derivative prepared from crystalline AA and purified by recrystallization from acetone-ethanol. Calculation of the concentration of DHAA in the original solution from which the derivative was prepared required, in addition, consideration of the molecular weights of DHAA and its derivative, appropriate dilution factors, and since the reaction was incomplete, the percentage of coupling of DHAA with DNPH. The latter was found to be 67.

Investigation of interference by reduced AA. Solutions containing between 0.08 and 0.25 mg. of reduced AA per ml. of citrate-phosphate buffer, pH 3.6 ± 0.1, were prepared. The lowest concentration was approximately 10 times that of DHAA at the midpoint of the standard curves established for the determination of DHAA. The reaction was carried out as described in the preceding sections except that concentrations of 0.25%, 0.5%, and 5.0% as well as 1.0% thiourea were used in the DNPH reagent.

Amounts of DHAA found after incubation ranged from 1.0 to 2.0% of the original concentration of reduced AA and were therefore sufficiently large to make the use of a correction factor advisable. The value selected for this factor, 1.4%, was the average obtained when concentrations of thiourea and reduced AA most closely resembled those existing in determinations on vegetable extracts (1% thiourea and 0.25 mg. reduced AA per ml.).

RESULTS AND DISCUSSION

Absorption spectra of DNPH derivatives. Typical absorption spectra of DNPH derivatives before and after chromatographing are shown in Figures 1 and 3; that of a purified derivative prepared from crystalline AA is shown in Figure 2 as a reference standard.

Clearly, the former which represent derivatives prepared under conditions of the usual DNPH method did not show the maxima at 370 and 530 mμ and minimum at 450 mμ typical of the standard preparation. On the other hand, one of the latter, that of the blue fraction, followed the general pattern of the reference compound, but showed a maximum at 530 mμ somewhat smaller in proportion to the maximum at 370 or the minimum at 450 mμ than did the reference compound. Such deviations may result in part from contamination with another fraction which moved down the column only slightly faster than the blue fraction and which could be separated from it only in an occasional sample. If the cases in which it could be separated (for example, cooked cabbage, Figure 3) are typical, however, its low absorption at 520 mμ suggests that its inclusion in the blue fraction would not lead to significant inter-

[c] The sum of the optical densities of the fractions expressed as a percentage of the optical density of the unchromatographed derivative. Recoveries were calculated for the wave lengths of the maxima and minimum of derivatives prepared from crystalline AA.

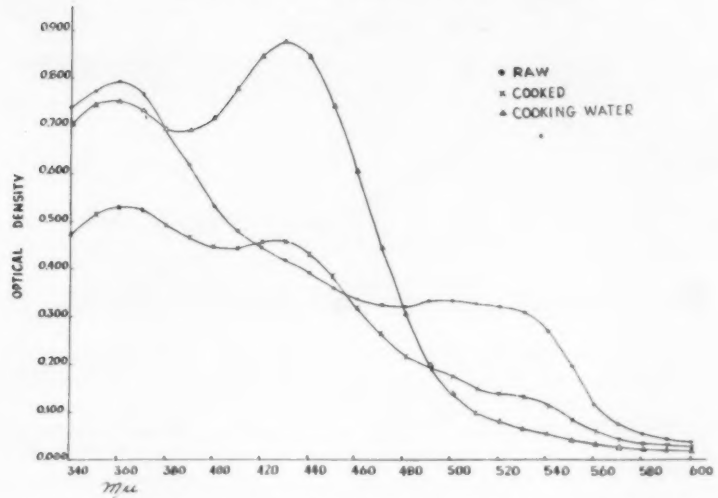

Figure 1. Absorption spectra of 2,4-dinitrophenylhydrazine derivatives prepared from unoxidized cabbage samples.

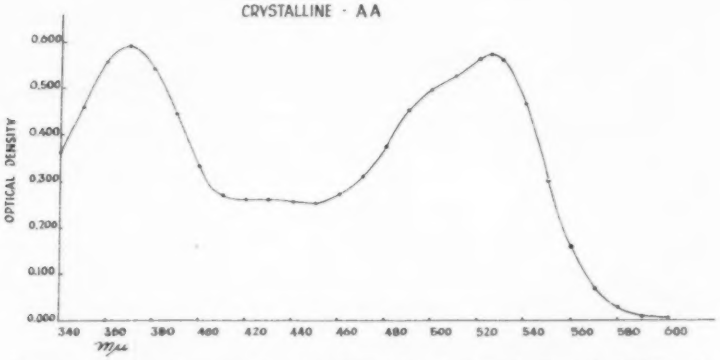

Figure 2. Absorption spectrum of 2,4-dinitrophenylhydrazine derivative of ascorbic acid.

ference in determinations of DHAA based on the optical density of the blue fraction at 520 mμ.

Spectra of other non-AA components represented by the various yellow fractions in Figure 3 showed either steadily decreasing optical densities over the entire range or a plateau or peak in the region of 420 to 440 mμ followed by steadily decreasing values. However, the absorption at 520 mμ of these fractions was a substantial part of the total absorption of the unchromatographed derivative at this wave length and might be expected to contribute to high values for DHAA when determinations are based on the unchromatographed derivative.

Concentrations of DHAA. Concentrations of DHAA as determined by the DNPH method with and without purification of the derivatives and with

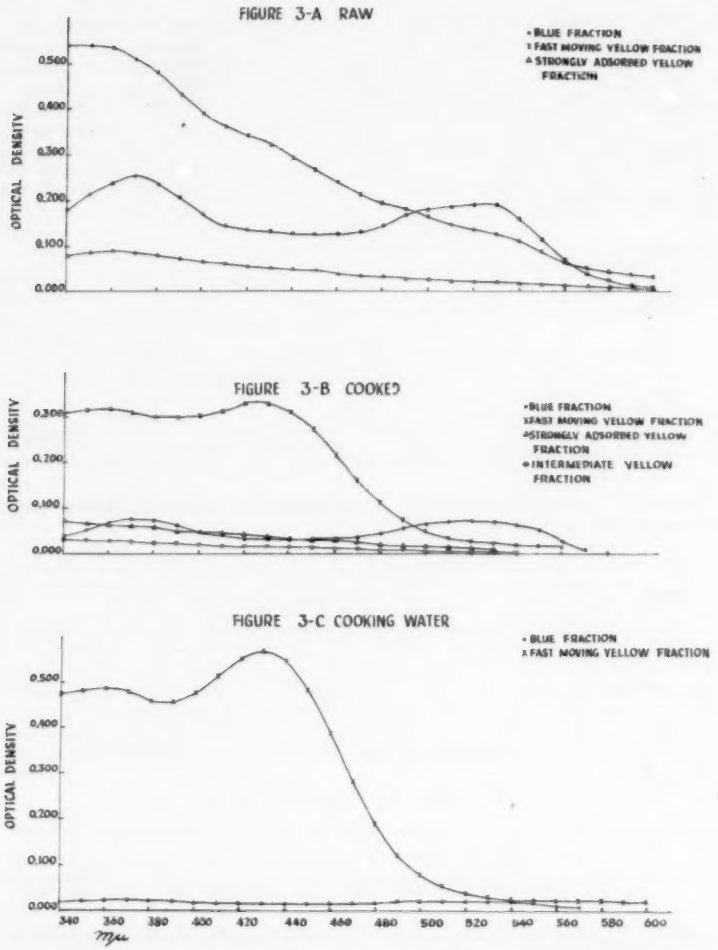

Figure 3. Absorption spectra of fractions separated from 2,4-dinitrophenylhydrazine derivatives prepared from unoxidized cabbage samples.

and without correction for interference by reduced AA are given in Table 1. In both vegetables, concentrations based on the purified derivatives were considerably lower than those based on the DNPH method. For example, in raw asparagus, they were only one-tenth and in raw cabbage, about one-half of the original value. Moreover, these concentrations were lowered still further when corrected for the interference of reduced AA. Thus, for the vegetables studied, non-AA components and reduced AA were responsible for an appreciable portion of the absorption at 520 mμ of the unpurified derivative.

SUMMARY

The suitability of the DNPH method for determining DHAA in raw and cooked cabbage and asparagus has been investigated by comparing concen-

TABLE 1

Concentration of Dehydroascorbic Acid in Cabbage and Asparagus, as determined by various methods (Mg. per 100 gm. vegetable).[1]

Sample	DNPH	DNPH purified derivative	DNPH purified derivative corrected for interference by reduced AA
Cabbage — Raw	4.2	2.2	1.7
Cooked	2.8	1.8	1.6
Cooking water	5.7	1.2	0.7
Asparagus — Raw	2.5	0.3	0.0
Cooked	1.3	—	—
Cooking water	2.6	1.2	1.0

[1] Cooking water concentrations expressed as mg. per total cooking water.

trations determined by the DNPH method with and without purification of the DNPH derivative. As part of the purification of the derivatives, chromatograms of the derivatives were prepared, absorption spectra of the derivatives before chromatographing and of the fractions separated by chromatographing were determined, and the concentration of DHAA was calculated from the optical density at 520 mμ of the AA fraction.

Absorption spectra of unchromatographed derivatives deviated greatly from the reference spectrum of a derivative prepared from crystalline AA. Those of the blue fraction which served as the basis for calculating the concentration of DHAA followed the general pattern of maxima and minimum typical of the reference spectrum, but the maximum at 530 mμ was smaller in proportion to the maximum at 370 mμ and minimum at 450 mμ than would be expected from the reference values.

Concentrations of DHAA based on the purified derivative and corrected for interference due to reduced AA were lower than those based on the DNPH method in both vegetables.

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SUITABILITY OF 2,4-DINITROPHENYLHYDRAZINE METHODS FOR DETERMINING DEHYDRO- AND TOTAL ASCORBIC ACID IN RAW AND COOKED VEGETABLES
II. TOTAL ASCORBIC ACID[a]

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(Manuscript received April 29, 1958)

Determinations of total ascorbic acid (AA) might be expected to be less affected by interference of non-AA components than direct determinations of dehydroascorbic acid (DHAA) simply because the concentration of total AA in vegetables is generally considerably greater than that of DHAA. On the other hand, they might be influenced by the action of various oxidizing agents on non-AA components if the absorption characteristics of the latter were altered.

In the present study, the specificity of the 2,4-dinitrophenylhydrazine (DNPH) method for determining total AA in vegetables was investigated by procedures already described *(3)*. Thus, DNPH derivatives were purified by chromatographic techniques, concentrations of total AA calculated from the fraction most closely resembling a reference compound prepared from crystalline AA, and these concentrations compared with those found by the usual DNPH methods.

METHOD

The DNPH method and chromatographic analysis of DNPH derivatives were carried out as described *(3)*, except that aliquots of the same vegetable extracts used for determining DHAA were treated with indophenol *(1)*, charcoal *(7, 9)*, bromine *(8, 9)*, or hydrogen sulfide followed by bromine *(8)* prior to adding the DNPH reagent. In addition, the concentration of thiourea in the DNPH reagent was reduced to 0.25%, the concentration suggested by Lowry, Lopez, and Bessey *(5)*, since preliminary studies with cabbage indicated that the same concentration of total AA was found in the presence of 1.0 and 0.25% thiourea.

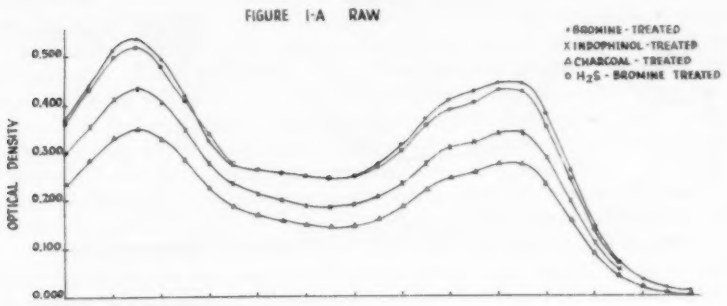
Standard curves were prepared from variously oxidized known solutions and were found to be essentially equivalent for the range of DHAA from 0.0015 to 0.0140 mg. per ml. Consequently, a single curve, drawn from the combined data, was used as the basis for calculating concentrations of DHAA in unknown solutions.

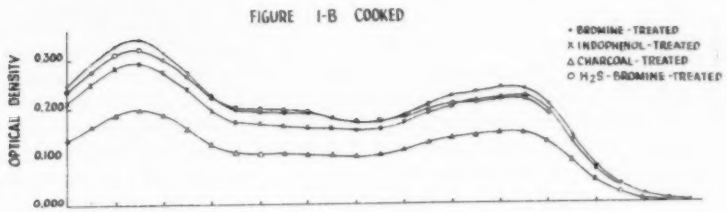
Data for concentrations of total AA and percentage retentions were subjected to analyses of variance, using a split plot design with preliminary treatments as main effects and methods of analysis as sub-effects. Duncan's multiple range test *(2)* was used to determine the significance of differences among means when significant values of F were found.

RESULTS AND DISCUSSION

Absorption spectra of derivatives before and after chromatographing. Absorption spectra of a typical series of DNPH derivatives before and after chromatographing are shown in Figures 1 and 2. Like these, the spectra of all of the unchromatographed derivatives failed to show the typical pattern of the reference spectrum of the derivative prepared from crystalline AA (Figure 2, Part I). Within each vegetable, cooking water samples deviated

[a] Scientific Journal Series Paper No. 3933, Minnesota Agricultural Experiment Station.





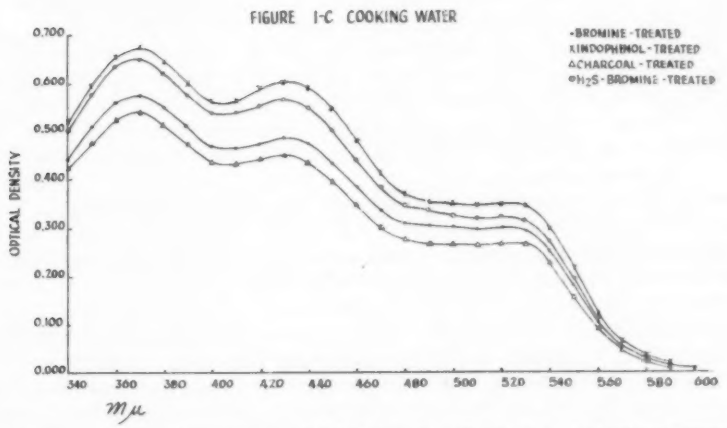


Figure 1. Absorption spectra of 2,4-dinitrophenylhydrazine derivatives prepared from cabbage, before chromatographing.

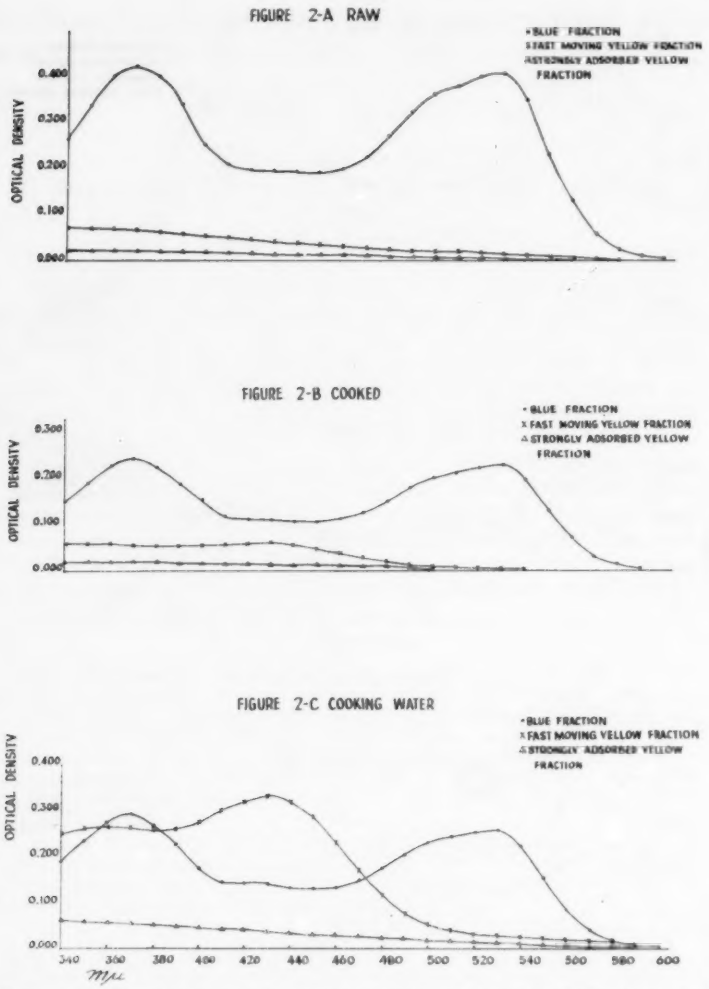


Figure 2. Absorption spectra of fractions separated from 2,4-dinitrophenylhydrazine derivatives prepared from bromine-oxidized cabbage.

from the reference pattern more than raw or cooked samples. In addition, bromine- and hydrogen sulfide-bromine-treated samples showed greater deviations than indophenol- and charcoal-treated samples. On the other hand, as judged by the ratios of the optical densities at the maxima and minimum, absorption spectra of the blue fractions separated by chromatographing were similar to that of the reference compound. For example, the ratios of optical densities at 530 mμ to those at 450 mμ were 2.11 and 2.15 for raw cabbage and the reference compound, respectively, and for the wave lengths 370 and

530 mμ, 1.08 and 1.05, respectively. Furthermore, the differences among the ratios for blue fractions from raw, cooked, and cooking water samples as well as those from variously oxidized cabbage samples were found to be non-significant at the 5% level. Thus, the chromatographic technique appeared to be an effective method of obtaining from dissimilar sources a characteristic fraction which resembled the reference compound very closely. The significance of this separation will now be considered in terms of the concentration and percentage retention of AA calculated from the optical densities at 520 mμ of this fraction and of the unchromatographed derivative. The wave length 520 mμ was chosen for these comparisons rather than 540 mμ, as suggested by Roe and Kuether *(7)*, because the optical densities of the non-AA fractions decreased less rapidly as the wave length was increased from 520 to 540 mμ than did the optical densities of the AA fraction. Therefore, the contribution of the former to the total optical density and consequently the interference would be greater at 540 mμ than at 520 mμ.

Concentration and percentage retention of total AA. Concentrations of total AA based on DNPH derivatives with and without purification are summarized in Table 1. Differences in concentration as determined with and without purification, although small in each case, were statistically significant in asparagus but not in cabbage. For asparagus, the concentrations in the raw and cooked samples and in two out of three replicate cooking waters were higher when calculated on the basis of the unpurified than the purified derivatives.

In many studies of methods of cooking vegetables, the important comparisons are concerned with the proportions of the original AA content retained in the cooked samples or the combined cooked and cooking water samples. Results shown in Table 2 indicate that if the amount of AA dissolved in the cooking water is disregarded and only that present in the raw and cooked samples considered, similar retentions would be found by the two methods in cabbage but not in asparagus. In the latter, percentage retentions based on the DNPH method without purification are higher than those based on the purified derivative. If the AA dissolved in the cooking waters is included, however, retentions based on the purified derivative are lower than those based on the DNPH method in both vegetables. Thus, the extent of agreement between the two methods of analysis was found to depend on the particular comparison being made and the vegetable in which the comparison was made.

The importance of the choice of oxidizing agent also appeared to depend on the specific purpose for which the determination was being made since some of the individual concentrations of total AA but none of the percentage retentions were affected by the preliminary treatment of the extract. For example, concentrations in the variously oxidized raw asparagus and cooked cabbage samples were found to be significantly different from one another. In the former, in which differences due to method of analysis have already been pointed out, those attributable to preliminary treatment lay within the group of unpurified rather than purified derivatives, the concentration in bromine-treated samples being greater than in charcoal-treated ones, and these in turn greater than in indophenol-treated ones. On the other hand, in cooked cabbage, in which concentrations based on purified derivatives were not different from those based on the unpurified derivative, the average concentra-

TABLE 1

Concentration of total ascorbic acid in vegetable samples found by DNPH method with and without purification

Preliminary treatment of extract	Concentration of total ascorbic acid					
	Raw (mg./100 gm.)		Cooked (mg./100 gm. raw wt.)		Cooking water (mg. per total cooking water)	
	Without purification	With purification	Without purification	With purification	Without purification	With purification
Cabbage						
Indophenol	31.0	40.5	16.9	20.4	33.4	35.7
Bromine	37.5	41.2	18.9	20.6	33.6	34.2
Charcoal	37.1	35.5	18.3	15.7	35.6	27.6
H_2S-Bromine	37.2	42.1	19.9	17.9	38.9	31.4
Mean	35.7	39.8	18.5	18.7	35.4	32.2
Difference between means	n.s.		n.s.		n.s.	
Asparagus						
Indophenol	30.5	29.3	19.5	14.2	19.2	16.1
Bromine	39.8	32.3	23.4	16.4	—	—
Charcoal	35.2	30.4	20.6	12.7	—	—
Mean	35.2	30.7	21.2	14.4		
Difference between means	*		*			

* — significant, n.s. — non-significant at 5% level.

TABLE 2

Percentage of total ascorbic acid retained in cooked cabbage and asparagus and in combined cooked and cooking water samples

Preliminary treatment of extract	Percentage of total ascorbic acid retained							
	Cooked cabbage		Cooked asparagus		Cooked cabbage plus cooking water		Cooked asparagus plus cooking water	
	Without purification	With purification	Without purification	With purification	Without purification	With purification	Without purification	With purification
Indophenol	55	51	70	55	99	89	104	82
Bromine	51	49	61	53	90	84	—	—
Charcoal	49	45	61	44	91	81	—	—
H_2S-Bromine	54	44	—	—	99	77	—	—
Mean	52	47	64	51	95	83		
Difference between means	n.s.		*		*			

* — significant, n.s. — non-significant at 5% level.

tion found by the two methods in bromine- and hydrogen sulfide-bromine-treated samples and in hydrogen sulfide-bromine- and indophenol-treated samples were similar to one another, but greater than in charcoal-treated ones.

Contrary to the work of Hewston, Fisher, and Orent-Keiles *(4)* with glucoreductone and of Mills and Roe *(6)* with orange juice, the various oxidizing agents produced no striking differences in the nature of the non- AA fractions, since the optical densities of these, like those in unoxidized extracts, either decreased steadily over the entire range of wave lengths or showed a plateau or peak in the region of 420 to 440 mμ. Differences among unchromatographed derivatives appear therefore to be due to variations in proportions of AA and non- AA components rather than to differences in the nature of non- AA components.

SUMMARY

Concentrations and percentage retentions of total AA in variously oxidized cabbage and asparagus samples have been determined by the DNPH method and by the optical density of DNPH derivatives prepared under the conditions of the DNPH method but purified by chromatographic methods.

Concentrations of total AA based on the purified derivative were significantly lower than those based on the DNPH method in asparagus but not in cabbage.

Percentages of total AA retained in the cooked sample based on the purified derivative were found to be significantly lower than those based on the DNPH method in asparagus but not in cabbage.

Percentages of total AA retained in the combined cooked and cooking water samples from cabbage based on the purified derivative were found to be significantly lower than those determined by the DNPH method. Percentages for asparagus showed a similar trend.

A general approach to decisions concerning the use of the DNPH method is suggested by these studies. This approach consists of isolating the DNPH derivative so that its properties can be compared with those of the derivative of crystalline AA. If differences are found their significance can be investigated by the chromatographic analysis for the purpose both of evaluating the contributions of component fractions to the total optical density of the unchromatographed derivative and of calculating the concentration of ascorbic acid from the optical density of the AA fraction separated by chromatography.

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A STUDY OF APPLE CELLULOSE[a, b]

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Cellulose, the most abundant of the naturally occurring organic substances, is apparently present in all plant tissues. Yet, disregarding the data on wood and cotton, which serve as sources of commercial cellulose, and a few crops of importance in animal feeding, little information is available on the proportion of cellulose in plant tissues. This scarcity of information is particularly evident with regard to fruits and vegetables, and it is disappointing since cellulose is likely involved in lending foods of plant origin their typical and desirable structure. Cellulose may also play a role in the behavior of various plant tissues as they undergo manufacture into food products.

There are a few older reports in which procedures not considered entirely reliable today have been used for the estimation of the cellulose content of apples *(2)*. Wehmer *(10)* states that apples contain 0.9% cellulose and Smock and Neubert *(8)* mention that the cellulose content of ripe apple flesh is about 1%. This latter figure appears in most tables of the proximate composition of food materials *(9)* which give the cellulose content in terms of "crude fiber" *(1)*, an empirical fraction known to include lignin and perhaps some other constituents in addition to cellulose.

In our studies of the effects of gamma radiation on plant tissues *(4, 6)* we have used a comparatively simple method for estimating and evaluating cellulose isolated from apples, carrots, and beets. The cellulose was characterized by its solution viscosity when dissolved in cupriethylene diamine (CED). This method was thoroughly tested on pure cotton and wood cellulose *(3)*. Any information which may throw light on the reasons for the texture and firmness differences and changes in apples would aid us in our studies of radiation effects. It seemed desirable, therefore, to take advantage of the large collection of apple varieties available at this Experiment Station for a preliminary study of the cellulose in apples of different varieties well characterized for their firmness and storage behavior. The present paper reports the results of cellulose determinations and evaluations on 17 varieties of apples[c] soon after picking and after storage. Although much remains to be done to clarify the nature of apple texture, we believe that the results of this brief investigation illuminate some of the factors involved and thus might interest others active in this field of research.

EXPERIMENTAL PROCEDURES

Preparation of alcohol insoluble solids (AIS) from apples. The freshly picked apples for this work were provided by the Pomology Department of the Experiment

[a] Approved by the Director of the New York State Agricultural Experiment Station, Geneva, as Journal Paper 1131, June 24, 1958.

[b] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

[c] We are indebted to members of our Pomology Department and particularly to Mr. Leo Klein for assistance in this investigation.

Station. Apples were picked at the normal harvest time for commercial storage and stored in brown paper bags in a cold room at 34°-36°F. (without atmosphere control) until used in the work reported here. This initial storage period was never more than 3 days. Then the apples were allowed to stand at room temperature for approximately 4 hours and weighed. The firmness of the apples was determined (after removal of the skin) with the Magness-Taylor tester *(7)*. Four firmness measurements were made on each of 4 apples of each variety. For the preparation of alcohol-insoluble solids (AIS) other apples were peeled, cut, and 300 g. of the cut sample not including core material was introduced into a beaker containing 600 ml. of hot 95% ethanol. The ethanol was brought to a boil and boiled 5 minutes. The level of the liquid was marked before boiling and made up to mark with 95% ethanol after boiling. Samples were covered and allowed to stand overnight at room temperature. Next day the samples were macerated for 5 minutes in a Waring blender using a rheostat for speed regulation. The ground samples were then transferred into a beaker using some 70% ethanol for rinsing and adding enough 70% ethanol to cover the sample.

After several days of standing, the samples were filtered through double layer of tightly-woven (80 x 120 fibers per inch) nylon cloth and the solids placed into 95% ethanol, covered, and left overnight at room temperature. Next day the ethanol was filtered off on double nylon cloth and the sample covered with absolute ethanol and allowed to stand overnight after which the liquid was again drained off and replaced by fresh absolute ethanol. Finally the sample was drained through double nylon cloth until the filtrate was clear, the solids spread out on nylon cloth on a glass plate and allowed to dry first at room temperature and afterwards in a desiccator in 100 mm. mercury vacuum over P_2O_5 for several days. Then the sample was ground in Wiley mill to pass a 30-mesh sieve and stored in a screw-top glass jar.

For the storage tests the apples were stored in bushel baskets at the above indicated temperatures.

Digestion of pectic constituents. For the removal of pectins, 0.250 g. of AIS was placed in a 100 ml. centrifuge tube and then 25 ml. of a filtered 1% solution of Pectinol 100 D was added together with a few drops of toluol. The mixture was frequently agitated to allow proper wetting. After 3 hours another 25 ml. of Pectinol solution was added and the mixture allowed to stand overnight at 30°C. Next day the sample was washed 3 times with water of about 80-90°C. and then twice with 95% ethanol and each time centrifuged to separate the solids. Finally the sample was centrifuged for 15 minutes at 2,000 r.p.m. in an International No. 2 centrifuge, the liquid poured off, and the sample dried first in air and then over P_2O_5 and stored in this form until used for cellulose extraction.

Cellulose extraction, evaluation, and recovery. Details of these procedures have been described elsewhere *(3)*. Briefly, solution viscosities were determined in cupriethylene diamine (CED) solvent at 25°C. using Ostwald-Cannon-Fenske viscometers. From the original solution a series of dilutions were prepared for viscosity measurements at lower cellulose conentrations. All CED solutions were collected and all the viscometers, pipettes, etc. were rinsed with water to make a total of about 250 ml. in order to recover all cellulose solution. The cellulose was precipitated from this solution by the dropwise addition of 27 ml. of 5 *N* H_2SO_4 under vigorous stirring. The precipitate was washed in a centrifuge tube several times with water and then with 95% ethanol, transferred into a previously weighed fritted glass crucible, and washed again with ethanol. Finally it was sucked dry, dried overnight at 95°C., and weighed. After calculation of the cellulose concentration in the various dilutions, the intrinsic viscosities were obtained by plotting specific viscosity/C as a function of concentration, C, and extrapolation to zero concentration. From the weight of the cellulose first the cellulose content of the AIS and then that of the fresh apple tissue were calculated.

When tested on pure cellulose *(3)*, this procedure gave 96-98% recovery. We have tested the possible undesirable effects of the Pectinol treatment on the cellulose but found that in spite of the reported presence of traces of cellulase activity *(5)*, neither the recovery nor the solution viscosities were affected under the conditions used. We have tested the reproducibility of the method on several samples. In one instance the intrinsic viscosity values of quadruplicate samples were 9.23, 8.48, 8.60, and 8.52; and the cellulose concentrations in the original solution were 0.153, 0.166, 0.158, and 0.160%, respectively. In a second set of quadruplicates on a different sample we obtained 8.52, 9.05, 9.08, and 9.19 for the intrinsic viscosities and 0.178, 0.172, 0.166, and 0.175% for the cellulose

concentrations. Attempts to refine the method were unsuccessful and since much closer results were obtained on pure cellulose samples, the variations were ascribed to the inhomogeneity of the AIS used as starting material.

A point of interest is that in dissolving the digested AIS residue in CED, invariably some CED-insoluble material was obtained. This resisted dissolution even upon the repeated addition of new CED. The proportion of this residue, which we have purified and dried, amounted to an average of 0.06% on the fresh apple tissue basis and ranged from 0.013% to 0.194%. Variations were found even among duplicate samples but the proportion of this residue was in no way related to the viscosity values obtained or the proportion of cellulose recovered. The proportion of this CED-insoluble fraction was somewhat higher in the stored apples than in the freshly picked ones.

RESULTS AND DISCUSSION

The experimental results obtained are presented in Tables 1 and 2. Our original plans called for a comparison of "hard cooking" and "soft cooking" varieties of apples, as classified by our Pomology Department. Such classification corresponded in all but one case to the original firmness values. The apple varieties used are listed in Table 1 in order of decreasing initial firmness as measured by the pressure tester. These values ranged from 25.5 to 11.3 lbs. As expected, all apples were softer after storage. The last column in the table shows the "per month" softening. In comparing the upper and the lower half of this column of figures, it is clear that the softening is more rapid in the apples which were firm at the time of picking than in the apples which showed lower initial pressure values.

The percentage of AIS in fresh apples ranged from 1.82% to 3.82% and in the stored apples from 1.99% to 3.72%. The initially firmer varieties contained a somewhat higher proportion of AIS than the softer apples but there are several exceptions to this relationship.

The percentage of cellulose in the apple flesh ranged from 0.29% to 0.69% in the freshly picked and from 0.30 to 0.67% in the stored fruit. The cellulose content decreased with decreasing initial firmness. As shown in Table 2, these two factors gave a coefficient of correlation of 0.796, significant at the 1% level. Calculation of the cellulose content on the AIS basis further accentuated the difference between the cellulose contents of the firm and soft varieties. On the whole, there was only a fair relationship between the cellulose content of fresh and stored apples but taking the upper and lower half of the column of figures, the stored firm apples showed a higher proportion of cellulose than the apples of the softer varieties. The average cellulose content of the fresh and stored apples was identical. Some ¾ of the cellulose percentage values on the stored apples were within 20% of the percentage contents of the fresh (unstored) apples of the same variety.

Taking the firmer apples as a group, the average intrinsic viscosity of the cellulose was higher here than the average for the values in the lower part of the table representing the varieties with lower initial firmness. However, this difference was irregular and the results were fairly inconsistent. As a result, the fact that firmer apples contain a cellulose of higher intrinsic viscosity, indicating higher average molecular weight, is discernable only when dealing with the results collectively rather than comparing the determinations on the cellulose from the individual varieties. The average intrinsic viscosity of fresh apples was somewhat higher than that of stored apples but on account of the variations this difference is not considered sig-

TABLE 1

Experimental results obtained on apples (1956)

Variety	Freshly picked						Stored at 34° F.						Firmness change per month in storage
	Picking date	Average weight of apples	Average firmness	AIS	Cellulose		Number of days in storage	Average weight of apples	Average firmness	AIS	Cellulose		
					In apple	Intrinsic viscosity					In apple	Intrinsic viscosity	
		g.	*lbs.*	%	%			*g.*	*lbs.*	%			*lbs.*
Stark [1]	10/24	178.0	25.46	3.69	0.62	9.33	131	169.5	20.21	3.29	0.61	8.97	1.20
Ben Davis [1]	10/25	181.9	22.98	3.82	0.69	9.25	135	164.0	17.53	3.72	0.67	10.12	1.21
Grimes [1]	10/31	128.0	22.14	3.13	0.52	9.01	131	132.3	15.91	3.03	0.50	8.88	1.43
Northern Spy [1]	10/31	272.7	21.96	2.82	0.51	10.23	131	241.9	17.20	2.07	0.34	8.66	1.09
Rome [1]	10/30	176.3	21.54	2.69	0.56	9.27	134	223.4	15.41	2.09	0.39	10.43	1.37
Baldwin [1]	11/1	257.9	20.28	2.41	0.48	8.61	120	259.0	15.21	2.07	0.37	7.96	1.27
Monroe [1]	10/12	186.8	19.78	2.04	0.44	9.70	132	172.3	16.84	2.62	0.52	9.46	0.67
Macoun [2]	10/9	152.9	19.53	1.96	0.29	8.72	104	154.6	13.78	2.08	0.32	8.98	1.66
20-Ounce [1]	10/12	258.9	19.21	2.82	0.45	10.38	103	299.1	11.04	2.49	0.49	9.45	2.38
Cortland [2]	10/16	194.5	15.66	2.24	0.37	9.36	102	238.4	14.04	2.30	0.40	8.42	0.48
Gravenstein [2]	8/25	168.2	15.41	2.43	0.43	9.42	105	165.2	12.72	2.31	0.40	8.40	0.77
Wealthy [2]	9/15	192.8	14.92	2.03	0.38	9.40	103	185.3	13.66	2.23	0.35	8.94	0.37
McIntosh [2]	10/10	200.6	14.72	2.07	0.36	9.36	105	200.5	11.15	1.99	0.39	7.92	1.02
Delicious [2]	10/10	245.1	13.46	2.22	0.32	8.53	131	146.3	12.34	2.11	0.40	8.49	0.26
Milton [2]	9/20	230.4	13.44	1.82	0.30	8.05	106	148.0	11.33	2.09	0.30	9.15	0.60
Franklin [2]	10/18	164.5	12.54	2.42	0.35	9.44	—	—	—	—	—	—	—
Fameuse [2]	10/24	140.4	11.29	2.64	0.39	9.32	102	126.7	10.08	2.43	0.46	8.68	0.36
	Averages:				0.438	9.24					0.431	8.94	

[1] Varieties usually designated as "hard cooking."
[2] Varieties usually designated as "soft cooking." According to Mr. Leo Klein, this Experiment Station, the Delicious apples in the present series appeared to be softer than in most seasons.

TABLE 2

Relationship between some properties and constituents of apples

	Coefficient of correlation
Firmness of (unstored) apples vs. percent cellulose	0.796 **
Firmness of (unstored) apples vs. percent AIS	0.716 **
Firmness of (unstored) apples vs. firmness change per month of storage	0.620 **
Cellulose percent vs. firmness change per month of storage	0.418 (NS)
Cellulose percent vs. "maximum days" storage life [1]	0.600 *

[1] See refer. *8*, page 14-15.
* indicates correlation significant at the 5% level.
** indicates correlation significant at the 1% level.
(NS) indicates that correlation is not significant.

nificant. At best, the difference is very small. The ranges of intrinsic viscosities were only 8.05 to 10.23 in the freshly picked apples and 7.72 to 10.43 in the stored apples. Values ranging from 6.50 to 8.90 were obtained in our unirradiated control apple samples in a separate recent study *(6)*. Using the same method of evaluation, cotton cellulose (filter paper) gave values around 4.5 and wood cellulose around 4.1 *(3)*. Since the results of intrinsic viscosity measurements give values which are directly related to the average degree of polymerization and thus the average molecular weight of cellulose, one may state that apple cellulose has a significantly higher average molecular weight than the two commercial celluloses tested.

Attempts to correlate the cellulose contents of apples of different varieties with their cold storage life expressed by Smock and Neubert in terms of "maximum days" (8, page 14-15) were only partially successful. The 11 varieties for which both data were available gave a coefficient of correlation of 0.600, significant at the 5% level. Only an indication of correlation was found between cellulose content and the order of the varieties according to their baking quality *(8,* page 20). However, these relationships might have been better with a larger number of comparisons and with cellulose measurements made on more than one set of samples as was done in the present study.

The main conclusion which may be drawn from this study is that whereas cellulose, particularly the proportion of cellulose, is likely to be a contributing factor in causing the firmness of freshly picked apples, changes which occur subsequently in the firmness are not caused by changes in the cellulose. The role of pectic substances in changes in the firmness of apples is well established but very little indication could be found, so far, that the initial firmness of apples is in any way directly related to total pectin content or the proportion of various pectic fractions (5). Although this is clearly an oversimplification of a complicated situation, one might state that generally speaking, the firmness of fresh apples seems to be closely related to the cellulose content whereas the softening is the result of pectic transformations which occur in the tissue.

SUMMARY

The firmness and cellulose content in the flesh of 17 varieties of apples have been determined when freshly picked for commercial storage. Similar determinations were made on apples of 16 varieties from the same lots after 102-135 days storage. The alcohol-insoluble solids (AIS) contents were also

determined in the course of cellulose isolation. Viscosity measurements were made on the isolated cellulose samples and their intrinsic viscosities determined.

Apples which are originally firm show more softening in storage than do apples which were not quite so firm. The cellulose contents of apples of different varieties were significantly different, those varieties known as "hard cooking" containing more cellulose than those known to be "soft cooking." The average cellulose content of the flesh of apples of 17 varieties was 0.438%. After storage, apples representing 16 of these varieties were found to contain an average of 0.431% cellulose.

The intrinsic viscosities of the cellulose samples isolated from "hard cooking" varieties averaged somewhat higher than the corresponding value for the "soft cooking" varieties. This difference was not clearly observable in comparing individual varieties. As compared with corresponding samples from the freshly picked apples, there was only a slight, if any, decrease in the intrinsic viscosity values of the cellulose isolated from stored apples.

These results are taken as an indication that cellulose concentration is a factor in apple firmness at the time of picking but that neither changes in the amount or quality of the cellulose are involved in the softening which occurs in storage.

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STUDIES OF PROCESSED ALL-GREEN ASPARAGUS. III.

QUALITATIVE AND QUANTITATIVE STUDIES OF NON-VOLATILE ORGANIC ACIDS BY CHROMATOGRAPHIC TECHNIQUES[a]

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Little is known of the organic acids present in processed all-green asparagus, or of the influence of harvest variables on their relative concentrations. Culpepper and Moon *(2)* reported in 1935 that as asparagus increased in height above ground the titratable acidity of the tip section of the spear increased. This increase in free acid was also reported in the first paper of this series on processed all-green asparagus *(3)*, and was shown to be accompanied by a decrease in hydrogen-ion concentration.

Preliminary paper chromatographic studies of asparagus brines indicated that as the asparagus matured, the concentration of citric acid increased rapidly in the 4-inch tip-section of the spears, whereas the concentration of malic acid appeared to remain constant. The presence of numerous other acids was also shown by these tests. A study was therefore undertaken to determine the organic acids present in processed all-green asparagus, and the influence of the height of asparagus at harvest, and the section of the spear selected on the organic acid content of the processed product.

EXPERIMENTAL

A. Qualitative Study of Acids

Asparagus strain 500 harvested at 3 to 6 in. above ground from plots on the University of California Campus, Davis, was used in this study. After washing, the 4-inch tip-sections were blanched in wet steam at 190°F. for 3 min. and then cooled in a water bath. These were drained for 3 min. and then packed in 211 x 400 cans with enameled ends. Each can contained 150 g. of asparagus and 150 ml. of distilled water. Cans were exhausted to a center temperature of 190°F., sealed and heat processed tips-up at 250°F. for 18 min. after a 5-min. come-up. Cans were cooled and stored under the prevailing laboratory temperature conditions.

To secure a high enough concentration of acid for analytical purposes, 5 cans were opened and treated as follows: The liquid was decanted and the spears combined and mixed with 750 ml. of 95% ethanol in a Waring blender for 5 min. The resulting slurry was filtered using suction. The asparagus solids and filter paper were blended with an additional 750 ml. of 95% alcohol and filtered using suction. The alcohol was removed from the combined filtrates by distillation under approximately 28 inches of vacuum. The aqueous solution obtained by this procedure was combined with the solution which had originally been decanted from the spears. This mixture was then passed through a Dowex 50 cation exchange column which was in the hydrogen form to remove metallic ions and amino acids. The acids in the effluent were then adsorbed on a Duolite A-4 anion exchange resin column which was in the hydroxide form. Each column was 2 cm. in diameter and 20 cm. long and was run at a rate of approximately 1 ml. of effluent per minute. They were operated so that the effluent from the cation column immediately passed onto the anion column. Sugars were removed by washing the columns

[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 28, 1958.

with 500 ml. of distilled water. Acids were eluted from the anion column with 2N NH_4OH. Elution with NH_4OH was stopped when the pH of the effluent became basic (as indicated by pHydrion paper). The column was washed with 250 ml. of distilled water and allowed to drain. The collected eluate was then passed through a Dowex 50 column to convert the ammonium salts to free acids. The cation column was then washed with 250 ml. of distilled water and allowed to drain. The effluent was concentrated to 5 ml. under reduced pressure.

Organic acids were separated by silicic acid column chromatography by the method of Bulen *et al (1)*. The column was prepared with 32 g. of silicic acid and 20 ml. of 0.5N H_2SO_4. Two grams of silicic acid were mixed with 2 ml. of the concentrated acid effluent and developed on the above column by elution with 100 ml. each of 5 and 10%; 200 ml. of 15%; 100 ml. each of 20, 25 and 30%; 500 ml. of 35%; and 300 ml. of 50% n-butanol in chloroform (v/v). Fractions of 5 ml. each were collected by an automatic fraction collector. They were titrated with 0.01N NaOH using one drop of 0.1% phenol red as an indicator. Fractions from the same titration peaks were combined and concentrated under vacuum to approximately 10 ml.

Each 10 ml. fraction was passed over a short Dowex 50 cation column to remove the sodium ions and then concentrated until a 0.01 ml. aliquot gave a satisfactory spot when paper chromatogrammed.

Organic acids present in each peak were identified by unidimentional descending paper chromatography on Whatman No. 1 paper. Three developing solvent mixtures were used: (a) n-butanol-acetic acid-water (4:1:5 v/v), (b) phenol-water-formic acid (75:25:1 v/v) and (c) 95% ethanol-water-conc. NH_4OH (8:1:1 v/v). The phenol for solvent (b) was purified by the procedure described by Draper and Pollard (4). Chromatograms were allowed to develop at 21°C. until the solvent front reached a position 1 in. from the bottom of the paper. Following drying, they were sprayed with a 0.04% solution of bromophenol blue in 95% ethanol at pH 5.8. Rf values were calculated from the center of the acid spot.

After tentative identification, the unknown was chromatogrammed adjacent to and mixed with the suspected known.

B. Quantitative Studies of Acids

1. Influence of height of asparagus at harvest. Asparagus of the Mary Washington variety was harvested from the randomized plots located on the University of California Campus, Davis. Spears 12 to 18 in. in height were harvested on April 30 and spears 3 to 6 in. and 6 to 12 in. in height on May 1. The spears were washed and the 4-in. tip-sections removed and graded as to spear diameter. Only those tips grading between ⅜ to ⅝ of an inch at the butt were used in this study. The tips within each height class were cut into one-inch sections and thoroughly mixed. One hundred gram portions of unblanched sections were placed in 303 glass jars with 350 ml. of distilled water. Jars were sealed with lacquered lids using a hand-operated steam-jet closing device. They were processed at 248°F. for 16 min. with 10 p.s.i.g. over-riding air pressure and cooled to 150°F. before releasing the pressure. Upon removal from the retort they were shaken to mascerate the tissues, allowed to air cool, and stored at room temperature.

A composite sample was prepared by blending the contents of 5 jars from each height class for 5 min. Insoluble tissues were removed by filtration using a small amount of Hy-flo Super-cel and suction. The filtrate was accurately measured and passed through the resin exchange procedure described previously except that Duolite C-3 in the hydrogen form was used as the cation exchange resin.

Neutral fractions passing through both columns were vacuum concentrated and made up to 100 ml. Acid fractions were vacuum concentrated and made up to 25 ml. The substances adsorbed on the cation columns were displaced with 2N NH_4OH and then vacuum concentrated and made up to 100 ml. Care was taken to keep all operations quantitative. Paper chromatograms of the anion, cation, and neutral fractions were prepared using solvent (a) to determine the completeness of the separation. The chromograms were sprayed with bromophenol blue.

Organic acids were quantitatively determined using a silicic acid column which was prepared from 16 g. of silicic acid and 10 ml. of 0.5N H_2SO_4. Three grams of silicic acid was mixed with 2 ml. of the concentrated acid obtained from the resin exchange column and developed on the column by elution with 100 ml. each of 15 and 20%, 200 ml. of 25%, and 500 ml. of 35% n-butanol in chloroform (v/v). Three ml. fractions were

collected and titrated as previously described. Each titration was corrected for the blank reading. The quantities of acid are reported as milliequivalents of acid per 100 g. of fresh weight. In this calculation it is assumed that an equilibrium exists between asparagus pulp and filtrate and that 1 ml. of filtrate represents 1 g. of the total contents of a jar.

Fractions from the same titration peak were combined, concentrated under vacuum to a few ml., and paper chromatograms were developed in solvents (a) and (c). After development, the papers were sprayed with bromophenol blue to determine the purity of the peaks.

2. Influence of section of spear selected for processing. Asparagus of the Mary Washington variety was harvested at 8 to 12 in. above ground for this study. Spears were washed and cut in four 2-in. sections from the tip downward. One hundred eighty-seven grams of each section was packed in a 303 glass jar with 225 ml. of distilled water. Jars were processed as described previously. Upon removal from the retort the samples were shaken to macerate the tissues and allowed to cool.

On opening, the insoluble tissues were removed by filtration through Whatman No. 1 filter paper. A 250 ml. portion of filtrate from each sample was passed over a Duolite C3 resin exchange column which was in the hydrogen form. Columns were washed with 500 ml. of distilled water and allowed to drain. The effluent was vacuum concentrated and made up to 50 ml. Paper chromatograms were developed in solvent (a) using 0.01, 0.02 and 0.04 ml. aliquots of the concentrates. After development, chromatograms were sprayed with bromophenol blue and visual observation was made as to the relative concentrations of acids present.

RESULTS AND DISCUSSION

Qualitative study of acids. The titers obtained for the various 5 ml. fractions eluting from the silicic acid column described previously are plotted in Figure 1. The major portion of the acidic constituents of asparagus is present in peaks 7, 9 and 10. The various peaks, labeled 1 through 11, were combined and concentrated as previously described. Results of the chromatographic analyses are presented in Table 1.

Rf values determined with known organic acids in the various solvents are shown in Table 2.

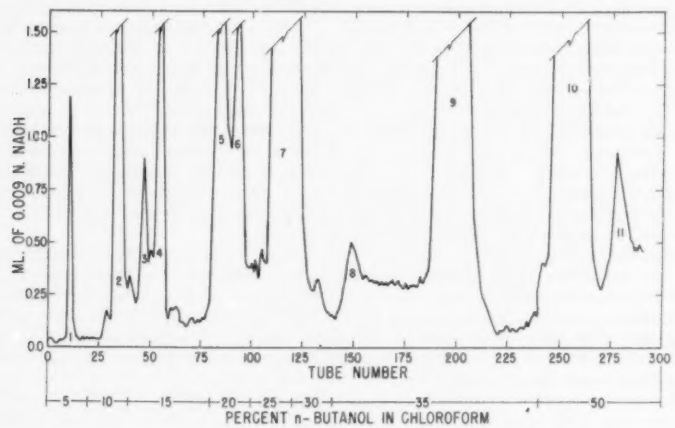

Figure 1. The separation of the organic acids of processed all-green asparagus by silicic acid column chromatography.

TABLE 1

Rf values and identification of unknown acids isolated from processed all-green asparagus

Peak No.	Solvent			Identification
	(a) n-butanol-acetic acid-water (4:1:5 v/v)	(b) phenol-water-formic acid (75:25:1 v/v)	(c) 95% ethanol-water-conc. NH_4OH (8:1:1 v/v)	
1	.34-.37	.05 & .80	.25 & .51	——
2	.80	.13 & .82	.17	——
3	.81	.46	.38	fumaric
4	.26	.22-.25	.45	——
5	.73	.54-.59	.33	succinic
6	.43	.26-.28	.34-.35	alpha-ketoglutaric
6	.71	.52	——	probably succinic
7	.58	.77-.80	.57	pyrrolidoxecarboxylic
8	.56-.58	.52	.50-.51	glycolic
8	.29	.26	.16	——
9	.49	.31	.23-.29	malic
10	.41	.15	.18	citric
11	.30	.36	——	——
11	.35	.03	——	——

TABLE 2

Rf values obtained for known organic acids in the solvents shown

Acid	Solvents		
	(a) n-butanol-acetic acid-water (4:1:5 v/v)	(b) phenol-water-formic acid (75:25:1 v/v)	(c) 95% ethanol-water-conc. NH_4OH (8:1:1 v/v)
Alpha-ketoglutaric	.43-.47	.28-.32	.34-.35
Citric	.37-.40	.15	.18 [1]
Fumaric	.84	.46	.38
Glutaric	.79-.80	.70	.34-.39
Glycolic	.59	.53-.54	.51
Malic	.49-.50	.31	.23-.29
Oxalic	.25	.24	——
Pyrrolidoxecarboxylic	.57	.78-.79	.56-.57
Succinic	.73-.74	.52	.32-.34

[1] Citric acid streaked in solvent (c) and did not give a distinctive spot.

Identification of the acids was based upon the similarity of their Rf values to that of a known acid in all 3 solvents, not only when chromographed in adjacent positions but when mixed with the known. Good agreement between knowns and the unknowns was obtained for fumaric, succinic, alpha-ketoglutaric, pyrrolidonecarboxylic, glycolic, malic, and citric acids. It is possible that peak No. 2 may contain glutaric acid and that the unidentified acid in 8 is oxalic. However, the samples isolated were not large enough for a more exhaustive study. Chelidonic acid, which has been identified in asparagus by Ramstad *(5)*, may also be one of the unidentified acids.

Pyrrolidonecarboxylic acid has been found in processed tomato juice by Rice and Pederson *(6)*. They observed that it was present only in the processed product and not in fresh tomatoes, and suggested that it might be formed from glutamine or glutaric acid during the processing. They also found that the concentration of this acid increased during storage of the canned product.

Chromatographic analysis revealed that pyrrolidonecarboxylic acid is not present in juice expressed from fresh asparagus. Thus, this acid appears to be produced during heat processing.

To determine its possible source, juice from fresh and processed asparagus were analyzed chromatographically in solvent (a). The paper chromatograms were sprayed with an 0.3% solution of nihydrine in 95% ethanol to develop the amino acids. Visual observation of the spots which developed on the chromatograms showed that the ratio of glutamine to the other amino acids was lower in the juice of the processed product, indicating a significant loss in this amino acid. The suggestion of Rice and Pederson that the pyrrolidonecarboxylic acid is derived from glutamine would seem to be born out in this case. No change in the relative concentration of any of the other amino acids was indicated.

Quantitative Study of Acids

1. Influence of height of asparagus at harvest. Results of the study on the influence of the height of asparagus at harvest on the organic acid content of the 4-inch tip-sections of processed all-green asparagus are presented in Figure 2. The millequivalents of acids are plotted against the midpoint of the range above ground at which the asparagus was harvested. Results confirm the visual observation of the paper chromatographic studies which indicated that the citrate content of the tip sections of asparagus spears increased as the asparagus increased in height above ground. Figure 2 shows that there is greater than a threefold increase in citrate content of the 4-in. tip-section as the spear matures from 3 to 6 in. to 12 to 18 in. above ground.

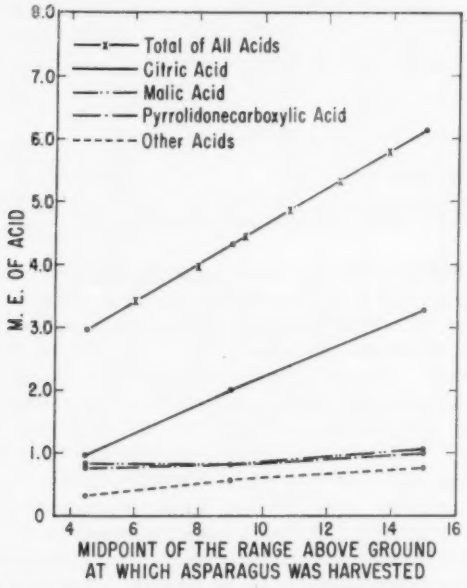

Figure 2. The milliequivalents of organic acid present in the processed 4-inch tip sections of asparagus harvested at 3 ranges of height above ground.

There appears to be a small increase in malic and pyrrolidonecarboxylic acids and a twofold increase in the total of all other acids emerging from the column. The change in ascorbic acid was described in a previous paper *(3)*.

An increase in pyrrolidonecarboxylic acid might be expected since as the asparagus matures the meristematic region becomes longer, which could probably result in an increase in the amino acid content of the 4-in. tip-section of the spear. Free-run liquid, drained from processed samples, and expressed from fresh spears, was analyzed chromatographically to check this point. The chromatograms were developed in solvent (a) and sprayed with a 0.3% solution of nihydrine in 95% ethanol. Visual observation of these chromatograms, however, indicated that only a very slight increase in the amino acid content of the tip-sections occurred as the spears increased in height above ground. This appears to explain the slight increase observed in pyrrolidonecarboxylic acid.

Acids present in the highest concentration are citric and malic. The ratios of citric to malic acid for the asparagus harvested at 3 to 6, 6 to 12, and 12 to 18 in. above ground were 1.10, 2.39, and 3.15, respectively. Extrapolation of the curves in Figure 2 would indicate that this ratio would be less than 1.0 for white asparagus. This was confirmed by visual observations of paper chromatograms of the brines from samples of canned white asparagus. Thus, the most dominant acid in processed asparagus is determined by the height above ground at which the spears are harvested.

Figure 2 also indicates a slightly more than twofold increase in the total milliequivalents of acid present in the 4-in. tip-section of the asparagus spear as it matures from 3 to 6 in. to 12 to 18 in. above ground. However, 5 strains of asparagus harvested from the same locality and handled and processed in an identical manner during the same season showed only a slight increase in titratable acidity and a decrease in hydrogen-ion concentration *(3)*. Comparison of these studies indicates that the major buffering system in asparagus is not the organic acids but is probably a combination of the acid anions, amino acids and proteins. This type of buffering system could explain the pH drop which normally occurs during the processing of asparagus. In the fresh asparagus the organic acids could be complexed with the proteins and amino acids and then partially released during the cooking by coagulation of proteins.

To determine the efficiency of the resin exchange procedure employed in obtaining samples for the above analysis the concentrated cationic, anionic, and neutral fractions were analyzed chromatographically with solvent (a). Results indicated that all of the organic acids studied were present only in the anionic fraction. Chromatograms of the concentrated eluates derived from the combination of fractions leading to pyrrolidonecarboxylic, malic, and citric acid peaks, were developed with solvents (a) and (c). These results showed that all peaks were pure except the malic acid peak for the sample prepared from asparagus harvested at 12 to 18 in. above ground. This peak was contaminated with an insignificant amount of citric acid, which probably contributed little to the overall titration value obtained for the malic acid. The silicic acid column procedure was performed in duplicate on the sample prepared from asparagus harvested at 3 to 6 in. above ground to check the reproducibility of the method. The results agreed within 2% for pyrrolidonecarboxylic and malic acids and within 7% for citric acid.

2. Influence of section of spear selected for processing. The influence of the section of the spear on the organic acid content of the processed product was determined by visual observations of paper chromatograms developed with solvent (a) and sprayed with bromophenol blue. At concentrations which yielded chromatograms with clearly defined spots and no streaking, compounds having Rf values of .84, .70, .53, .48, .40 and .20 were observed. The malic acid content appeared to be identical in all sections of the spear studied. All other acids decreased in concentration as the sections approached the butt end of the spear. Citric and pyrrolidonecarboxylic acids appeared to be 3 to 4 times higher in concentration in the tip section than in the lowest 2 in. section.

Chromatograms developed with solvent (a) and sprayed with the ninhydrin solution indicated that the amino acids were concentrated in the tip 2 in. sections and decreased rapidly in concentration in the lower sections. The difference in the glutamine content of the various sections could explain the difference in pyrrolidonecarboxylic acid content. It is also interesting to note that the hydrogen-ion concentration is lowest in the tip section of the asparagus spear *(3)*, yet the organic acid content is highest. This is considered as further evidence that the buffering system in asparagus is greatly influenced by the amino acids and proteins.

The influence of the age and distribution of the chladophylls on the chemical composition of the samples analyzed in this study was not investigated.

SUMMARY

Organic acids present in processed all-green asparagus were separated into various fractions by silicic acid column chromatography. The acids present in these various fractions were then identified by paper chromatographic techniques employing three developing solvents. The acids found were, fumaric, succinic, alpha-ketoglutaric, pyrrolidonecarboxylic, glycolic, malic and citric. The presence of other acids was indicated. Pyrrolidonecarboxylic acid was not found in fresh asparagus, and evidence indicated that it may be produced during heat processing from glutamine.

The influence of the height of asparagus at harvest on the organic acid content of the 4-inch tip-sections of processed all-green asparagus was studied by a procedure similar to that described above. A three-fold increase in citrate was found as the height of asparagus at harvest was increased from 3 to 6 inches to 12 to 18 inches. There was a small increase in malic and pyrrolidonecarboxylic acids and a twofold increase in the total of all other acids eluting from the column. The ratio of citric to malic acid changed rapidly as the asparagus increased in height above ground. Results indicate that the major buffering system in asparagus is not the organic acids but probably a combination of acid anions, amino acids and proteins.

The influence of the section of the spear on the organic acid content of the processed product as determined by visual observations of paper chromatograms revealed that the malic acid content was nearly constant in all sections studied. All other acids studied decreased in concentration as the sections approached the butt end of the spear.

Acknowledgment

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STUDIES OF PROCESSED ALL-GREEN ASPARAGUS. IV. STUDIES ON THE INFLUENCE OF TIN ON THE SOLUBILITY OF RUTIN AND ON THE CONCENTRATION OF RUTIN PRESENT IN THE BRINES OF ASPARAGUS PROCESSED IN GLASS AND TIN CONTAINERS*

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The approximate solubility of rutin in water has been reported by Griffith *et al. (3)*. The solubility curve for rutin in aqueous solution increases sharply at temperatures above 75° C. Below this temperature, rutin is only slightly soluble in water. The solubility characteristics of rutin explains why a rutin precipitate occurs when all-green asparagus is processed in glass containers; a large amount of rutin dissolves into the brine during the retort operation because of the high temperature, but the brine becomes saturated as the product cools and rutin precipitates.

A rutin precipitate is seldom observed in asparagus processed in a tin container. Comparison of the visual color of brines of asparagus processed in glass and in tin containers reveals that the former is a very light yellow while the latter is a brilliant yellow. The brilliant yellow is due to a tin-rutin complex formed from the tin which is etched from the container during the cook. This complex is similar to the aluminum-rutin complex which is used as a quantitative measure of rutin *(1)*. This suggests that the formation of the tin-rutin complex prevents the precipitation of rutin. A tin-coated lid has been used for some time by asparagus processors who pack in glass as it tends to result in a product of superior color as compared with that of asparagus packs processed with a lacquered lid. However, there are still frequent instances when glass packs with tin-coated lids contain a large amount of rutin precipitate.

When the authors were developing a quantitative method of analysis for rutin, a preliminary study was conducted to determine the influence of the stannous ion on the rutin content of asparagus brines. All-green asparagus of uniform size and maturity was processed in glass using lacquered lids. To one-half of the jars was added 100 p.p.m. of stannous ion as $SnCl_2.2H_2O$. The remaining jars were used as control. It was observed that the rutin content of the brines, as determined by a method to be described later, remained constant in the controls but was much lower in the samples containing the stannous ions and showed a tendency to decrease on storage. Thus, the rutin present in the brines of processed asparagus appears to decrease on storage in the presence of stannous ions. This suggests another possible reason for the failure of a rutin precipitate to form in asparagus which is processed in a tin container.

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* Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 28, 1958.

EXPERIMENTAL

1. Influence of the stannous ion on the procedure used for the analysis of rutin. Six hundred mg. of rutin were dissolved by heating in 1.75 liters of an 0.1M acetate solution, buffered at pH 5.2 with NaOH. After the solution had cooled, it was diluted to 2 liters with an 0.1M acetate solution buffered at pH 5.2, and 200 ml. of this solution was placed in each of 10 eight-ounce baby food jars. A one ml. aliquot of a solution containing 20,000 p.p.m. of stannous ions as $SnCl_2.2H_2O$ in an 0.1M acetate buffer at pH 5.2 was added to five of these jars. Therefore, the resulting solution contained approximately 100 p.p.m. of stannous ions. Jars were steam-jet closed using lacquered lids, retorted at 250°F. for 15 minutes using 10 p.s.i.g. of overiding air pressure and pressure cooled. Samples with and without the stannous ions were analyzed for their rutin content immediately after removal from the retort and after 1, 2, 5 and 10 days of storage at room temperature.

The rutin content of the samples was determined by a modification of the procedure described in a previous paper *(1)*. Aliquots of 0.5 ml. of rutin solutions were mixed with 3.5 ml. of the $AlCl_3$ solution buffered at pH 5.0 and allowed to react for 15 min. The optical density of these solutions was plotted against a blank of the buffered $AlCl_3$ solution on a Model DR Beckman spectrophotometer between 412 and 416 mμ. The peak reading was used as indicative of the rutin concentration. The optical densities were also determined in the usual manner against a blank of 0.5 ml. of the rutin solution in 3.5 ml. of acetate buffer.

2. Influence of the stannous ion on the solubility of rutin. Three series of rutin solutions were prepared in 8-ounce baby food jars in 200 ml. of an 0.1 M acetate solution which was buffered at pH 5.5. The rutin was weighed and added to each jar. The first series contained 30, 35, 40, 45, 50, 55, 60 and 70 mg. of rutin per jar. The second series contained approximately 100 p.p.m. of stannous ions as $SnCl_2.2H_2O$ in addition to the rutin. The third series contained 1 g. of tin metal in addition to the rutin and consisted of jars containing only 35, 40, 45, 50 and 55 mg. of rutin.

All jars were steam-jet closed with lacquered lid, and retorted at 250° F. for 15 min. with 10 p.s.i.g. overiding air pressure and pressure cooled. They were stored at room temperature and observed visually at different time intervals for a rutin precipitate. After 4 weeks of storage the jars were opened and the contents filtered through Whatman No. 1 filter paper by gravity. The rutin content of a four-fold dilution of the filtrate was determined by the method previously described in which the spectra was obtained using buffered $AlCl_3$ as a blank.

3. Influence of processing asparagus in glass or tin containers on the concentration of rutin in the brine. Asparagus, strain 500W, was harvested at 6 to 12 in. above ground from plots on the University of California Campus at Davis. The spears were washed and the 4 in. tip section removed. Tip sections with base diameters of ⅜ to ⅝ in. were used in this study. They were blanched in an atmosphere of steam at 195° F. for 1 minute and allowed to air cool. Then they were cut into 1-in. sections, thoroughly mixed and 75 g. of the mixture was packed with 125 ml. of distilled water in 8-ounce baby food jars. Forty-eight jars were prepared in this manner. Twelve jars with lacquered lids were immediately steam-flow closed to serve as controls. A one-ml. aliquot of a solution containing 20,000 p.p.m. of stannous ion as $SnCl_2.2H_2O$ was added to 12 of the remaining jars resulting in a stannous ion concentration for each jar of approximately 100 p.p.m. These jars with lacquered lids were steam-flow closed. The 24 remaining jars were steam-flow closed using 1¾ pound tin coated lids. Twelve of these jars were retorted lids-down; all other jars were retorted lids-up. A water cook at 250°F. for 16 min. with 10 p.s.i.g. overiding air pressure was used; the jars were water cooled to 150°F. under pressure.

Eight 211 x 400 cans with enameled ends containing 105 g. of asparagus and 175 ml. of distilled water were also prepared. These cans were exhausted for 5 min. at 212°F., closed hot, retorted at 250°F. for 16 min. after a 5 min. come-up, and water cooled.

All samples were stored at room temperature. At varying intervals of time, a container of each sample was opened. The pH of the brine of each jar was determined with a Beckman Model H2 pH meter. The solid material was separated by filtration using suction. Filter paper and solids were frozen for further analysis. Rutin content of an 0.5 ml. aliquot of the brine was determined by the $AlCl_3$ method described previously using the buffered $AlCl_3$ solution as a blank. Optical density of a 0.5 ml. aliquot of the

control sample in 3.5 ml. of pH 5.0 acetate buffer was also determined against a blank of buffered $AlCl_3$ solution.

Rutin content of the solids was analyzed as follows: Solids and filter paper for each sample was blended for 3 min. in 200 ml. of a 1.0% versene solution. The resulting slurry was heated in a glass covered container for 30 min. in a boiling water bath. Then the slurry was filtered hot through a thin layer of Hyflo Supercel. The filtrate was cooled and analyzed for its rutin content.

RESULTS AND DISCUSSION

1. Influence of the stannous ion on the procedure used for the analysis of rutin. Results of the analysis of the rutin content of samples containing 30 mg. of rutin per 100 ml. of solution with and without 100 p.p.m. of stannous ion are presented in Figure 1. These results show little difference in the optical density of the control samples containing 100 p.p.m. of stannous ions when their optical density were determined by the $AlCl_3$ method against a blank of buffered $AlCl_3$ solution. This shows that the stannous ion does not interfere with the above method of analysis for rutin, and suggests that the aluminum ion is present in sufficiently high concentration to displace the stannous ion in the tin-rutin complex. Also this method appears to give fairly consistent results over the 10-day period.

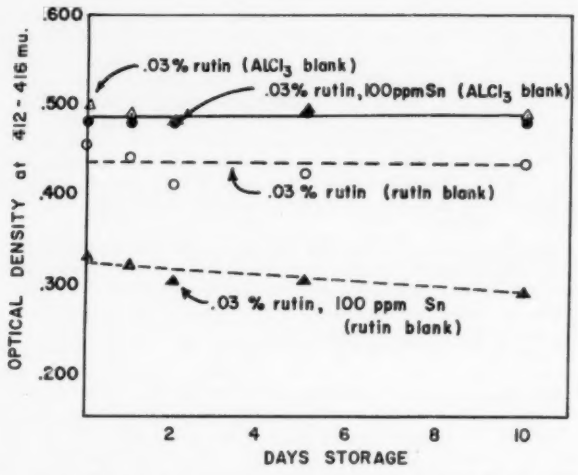

Figure 1. The influence of stannous ions on two methods of analysis for rutin over a period of time.

When the optical density of an 0.5 ml. aliquot of sample in 3.5 ml. of buffered $AlCl_3$ solution is determined against a blank of a similar aliquot in 3.5 ml. of buffer, it is displaced due to the optical density of the blank. This displacement is much larger in the sample containing the stannous ion due to the increased optical density of the blank caused by the presence of the colored tin-rutin complex. Because of this interference, all subsequent results presented in this paper are based on the use of buffered $AlCl_3$ solution as a blank unless otherwise specified.

Results for an individual treatment are within ± 5% of the average results for the treatment. This is not considered a significantly large variation. Also the results do not indicate any significant trends in changes in the concentration of rutin over the 10-day period of storage.

2. Influence of the stannous ions on the stability of rutin. The rutin was completely dissolved during the heat processing in all of the samples prepared for this study. Control samples were a light yellow in color and the samples treated with $SnCl_2.2H_2O$ were a very bright yellow and cloudy. The cloud was attributed to the formation of $Sn(OH)_2$. The samples containing 1 g. of tin metal per jar were only slightly brighter yellow than the controls indicating that only a small quantity of stannous ions entered solution during the cook.

The number of days required for the initiation of a rutin precipitate in the various samples is tabulated in Table 1:

TABLE 1

The number of days required for the initiation of a precipitate of rutin to be observed visually in an 0.1 M acetate solution of various concentrations of rutin buffered at pH 5.5

Treatment	mg. of rutin per 100 ml.							
	15	17.5	20	22.5	25	27.5	30	35
Control	*—	5	5	2	2	3	5⁄8	5⁄8
Control + 100 ppm Sn **	—	—	—	—	—	—	—	—
Control + 1 gm tin metal/jar	—	—	4	6	3	3	not run	not run

* Indicates no precipitation

Only 15 hours was required before a precipitate was observed in the control samples containing 35 and 30 mg. of rutin per 100 ml. of solution. The time required for the initiation of a precipitate in the control samples increased as the concentration of rutin in solution decreased; a precipitate did not occur in the samples containing 15 mg. of rutin per 100 ml. even after 3 months' storage. All of the samples containing 100 p.p.m. of stannous ions as $SnCl_2.2H_2O$ contained a very fine precipitate which was probably $Sn(OH)_2$; the formation of crystals of rutin was not observed. All of the samples containing 1 g. of tin metal per jar contained a rutin precipitate except the samples containing 17.5 mg. and 15 mg. of rutin per 100 ml. of solution.

The foregoing results indicate that the presence of stannous ions prevents the precipitation of rutin; that is, the tin-rutin complex is more soluble in the buffered solution than pure rutin. They also confirm the observation that only a small quantity of tin dissolved during the heat treatment.

After 4 weeks of holding at room temperature, all samples were opened and filtered by gravity through Whatman No. 1 filter paper. The optical densities at 412-416 mμ of 0.5 ml. aliquot of a 1 to 4 dilution of the filtrate as determined by the $AlCl_3$ procedure described previously are presented in Figure 2. All of the control samples having a rutin precipitate had optical densities far below that of the sample containing 15 mg. of rutin per 100 ml. of the acetate buffered solution. The approximate solubility of rutin under the conditions of this study was 8 to 9 mg. of rutin per 100 ml. of solution (O.D = 0.150). The

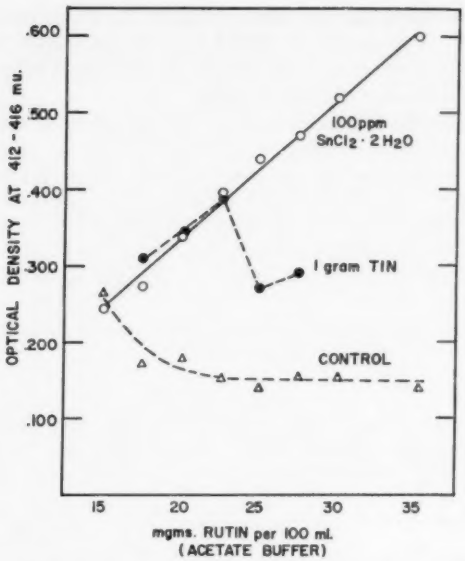

Figure 2. The influence of stannous ions on the solubility of rutin in 0.1 M acetate solution buffered at pH 5.5.

control sample which did not precipitate was apparently super-saturated. The optical density of the filtrates of samples containing 100 p.p.m. of stannous ions were proportional to the original rutin concentration showing that the tin-rutin complex is more soluble in the aqueous acetate buffer than pure rutin. Within the limits of this test, the solubility was increased four-fold over that of the solubility of pure rutin by the presence of 100 p.p.m. of stannous ions. The samples containing 1 g. of tin-metal per jar had a higher soluble rutin concentration than of the controls. The intensity of the yellow color of all samples in this series had increased over the 4-week period indicating that stannous ions were entering the solution. The jars containing the two highest rutin

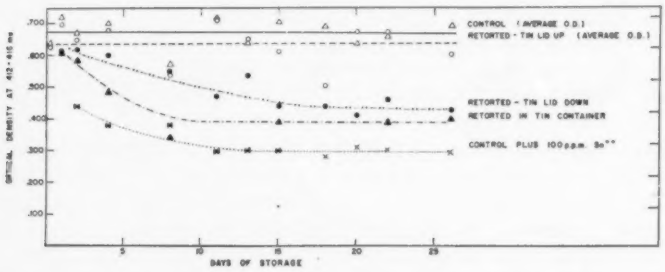
CONTROL (AVERAGE O.D.)
RETORTED - TIN LID UP (AVERAGE O.D.)
RETORTED - TIN LID DOWN
RETORTED IN TIN CONTAINER
CONTROL PLUS 100 p.p.m. Sn++
DAYS OF STORAGE

Figure 3. The change in rutin concentration of the brines of asparagus packs processed in different containers or stored in different positions as determined by the optical density.

concentrations were a lighter yellow in color than the other samples in this series and contained less soluble rutin. The less intense yellow color of these samples suggests that they contained a lower concentration of stannous ions which would result in a lower concentration of soluble rutin. It appears that it is essential for the stannous ions to be dissolved during the heat processing or soon thereafter, in order to prevent the initiation of a precipitate.

3. Influence of processing asparagus in glass or tin containers on the concentration of rutin in the brines. The optical density of 0.5 ml. aliquots of the brines read against a blank of buffered $AlCl_3$ solution for the various samples packed for this study are presented in Figure 3. Although the optical densities are not exact measures, they are excellent relative measures of the rutin concentration of the brines. Figure 3 shows that the rutin concentration of the brines of the control samples did not change over the 26-day period of this study. This also suggests that equilibrium is reached during the cook. The samples packed in jars with tinned lids and retorted lids-up have a slightly lower average optical density than the control samples and the optical densities of two of the solutions are much lower than the average. This is explained by visual observations of the lids from these jars; the samples having the low optical densities came from jars whose caps showed some corrosion of the tin at the edge of the lids. Thus, some tin was solubilized. All other treatments in this study resulted in a rapid decrease in the rutin concentration of the brines for the first 15 days of storage. Further storage did not seem to result in a continued decrease. The rate of decrease of the rutin concentration seems to be related to the apparent tin to product ratio. The rutin concentration in the brine decreased more rapidly in the samples processed in tin containers than in the sample processed in glass with tinned lid-down and stored lid-down. The decrease in optical density or rutin concentrations of the brines for the samples processed in glass with the tinned lids down and retorted with lids down was 34 percent, samples processed in tin containers showed a decrease in rutin of 42 percent and samples processed in glass with 100 p.p.m. of stannous ions and lacquered lids decreased 55% in rutin concentration. In this experiment, the product to brine ratio was much lower than in commercial packs of asparagus. This procedure was employed so that the brines of the controls would not become super-saturated during the heat processing and then precipitate rutin upon cooling and storage. If this had occurred, there would have been a considerable decrease in the rutin concentration of the brines of the controls during storage, rather than the constant concentration observed in the experiment.

The pH of all samples was 5.9, except those containing 100 p.p.m. of stannous ions which was 5.7. Also the optical density of the 0.5 ml. aliquot of the control in buffer determined against the buffered aluminum chloride solution remained constant over the entire period of the experiment and averaged .053.

Since the first phase of this study showed that stannous ions do not interfere with the procedure for the analysis of rutin used in this study, it appeared that the rutin migrated into the spear during storage by some mechanism which involves the stannous ion. However, since the first phase of this study was conducted on rutin solutions containing the asparagus, it was thought possible that in a complex solution like asparagus brine, stannous ion could

interfere with the rutin analysis. To check this point, the filtrate from several jars of asparagus which were processed with lacquered lids was thoroughly mixed. The optical density of an 0.5 ml. aliquot of this solution in buffered $AlCl_3$ solution was determined. Then 100 ml. aliquots of the filtrate were reprocessed in 8-ounce glass jars using tinned lids. Samples were retorted lid-down and upon removal of the jars from the retort, the solutions were a brilliant yellow showing the formation of the tin-rutin complex. Optical densities of these samples remained constant over a 10-day period indicating that the asparagus spears must be present for the rutin concentration of the brines to decrease.

The solid materials were then analyzed for their rutin content. An 0.5 ml. aliquot of the versene extract in buffered $AlCl_3$ solution was read against an 0.5 ml. aliquot of versene extract in acetate buffer. This procedure appeared to be satisfactory as the versene concentration was sufficiently high to prevent color development in the blanks. In the buffered $AlCl_3$ samples, the concentration of aluminum was sufficiently high to saturate the versene and further to react with the rutin. However, formation of the aluminum-rutin complex, as observed by the rate of color development, was slower than in samples which do not contain versene. Optical densities of the various versene extracts are presented in Table 2. The optical density of the sample processed in tin was corrected to a 75 g. basis. It is obvious from the table that the spear solids from samples having a source of tin, except the sample retorted tin lid-up, are higher in rutin concentration than the control. The brines show equivalent decrease therefore the rutin migrates back into the spear by some mechanism involving stannous ions. This is further supported by the fact that the initial samples were shaken to mascerate the spears and the filtrate of those samples containing stannous ions contained rutin concentrations approximating those obtained for samples containing whole spears after 15 days of storage. This is attributed to the increase in binding area of the spear solids due to maceration. It has also been observed that grinding asparagus spears in buffered $AlCl_3$ solutions will cause a binding of the rutin with the spear solids. Thus, this reaction may possibly occur with many polyvalent ions.

Upon removal of samples from the retort, it was observed that the edges of the chladophylls of the asparagus sample containing 100 p.p.m. of stannous ions were a very dark gray in color. The chladophyll contains a high con-

TABLE 2

The change in rutin concentration of the solid portion of asparagus packs processed in different containers and under different conditions as determined by the optical density of 0-5 ml. aliquots of a versene extract of the solids in buffered $AlCl_3$ solution against a blank of a similar aliquot of extract in acetate buffer. The optical densities were measured at 412-416 mμ.

Sample	Days of storage							
	4	8	11	13	18	20	22	26
control	.330	—	—	.370	—	—	.342	—
retorted tin lid-up	.300	—	.392	—	.322	.360	.355	.330
retorted tin lid-down	.374	.413	—	.382	.432	.419	.392	.405
retorted in tin container [1]	.362	.394	—	—	.405	—	.436	—
control + 100 ppm Sn ++	—	—	—	.500	—	—	.495	—

[1] Reading corrected for weight of stalk.

centration of anthyocyanin and it is believed that this dark coloration is due to a tin-anthocyanin complex formed in this area of the spear. Brines of these same samples also were darker in color than the sample of the various other treatments. The discoloration of the brine decreased with length of storage. The authors believe that the dark tin-anthocyanin complex may partially cause the dark coloration observed in the brines immediately after processing, for both white and green asparagus processed in tin containers. This particular discoloration of the brine disappears after several days of storage. The authors have also noted that this discoloration (observed the day after heat processing) is related to blanch temperature. Steam blanches of 190° F. for 1 minute almost completely eliminate this type of discoloration.

Results of this study clearly demonstrate that rutin does not precipitate in the brines of asparagus processed in a tin container due to the solubilizing action of the tin. The stannous ions greatly reduces the concentration of rutin in solution by binding the rutin in the spear solids and may also form a complex with rutin which is more soluble than pure rutin. This would suggest that the tin concentration of asparagus spears processed in tin containers or with a source of tin would be higher than that of the brines.

It appears that it is possible to prevent the precipitation of rutin in asparagus processed in a glass jar by processing with tinned lid down. Heat processing such samples lid-up does not appear to result in sufficient solubilizing of tin to cause an appreciable decrease in the rutin concentration of the brines, even if these samples are stored lid-down following processing. The tin coating of lids from jars which were heat processed lid-down are etched, whereas those from samples heat processed lid-up and stored lid-down appear normal except for some slight etching near the edge of the cap occasionally occurs.

Two other factors which have not been studied but are obviously of importance in preventing a precipitate in processed asparagus brines are: the ratio of tin surface to product, and the influence of compounds which may tend to protect the surfaces subject to detinning, such at the tin-oxide film that forms on tin plate with age. The influence of harvest variables and blanch and storage treatment on the rutin concentration of the jars were presented in previous papers *(1, 2)*.

SUMMARY

The effect of stannous ions on the solubility of rutin in aqueous solutions was studied in model systems and processed a green asparagus. The addition of 100 p.p.m. of stannous ions to a aqueous solution of rutin prevents its precipitation at concentrations 4 fold higher than its normal solubility. In the presence of asparagus, tin also increased the concentration at which precipitation will occur, however the presence of the spear solids exerts an adsorption effect on the tin rutin complex. The concentration of rutin in the processed spear increases during storage while the concentration of rutin in the brine decreases.

A method was developed for the analysis of rutin in the presence of tin in brines and the method modified to allow the determination of rutin in asparagus spear solids processed in the presence of tin.

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INHIBITION OF SULFHYDRYL ENZYMES WITH SORBIC ACID

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Gooding *(11)* found that certain of the α, β -unsaturated monocarboxylic aliphatic acids, including sorbic and crotonic acids, are good fungistatic agents for foods and food wrappers. Since then a great deal of work has been done on the toxicity, purity, method of determination, fate, and the use of sorbic acid as a fungistatic agent in such foods as cheese, fish, pickles, gum and sugar solutions, citrus products, fruit purees and pharmaceutical preparations. These investigations have been reviewed by Melnick, Vahlteich, and Hackett *(18)*. Deuel and Alfin-Slater *(3)* reviewed the advantages of using sorbic acid rather than sodium benzoate in foods. Emard and Vaughn *(4)* and York and Vaughn *(31)* have investigated the selective nature of sorbic acid on the catalase negative lactic acid bacteria and clostridia.

Some postulations have been presented to explain the mechanism of the inhibition of microbial growth by sorbic acid *(5, 17)* but no experimental work has been published on this. Mukherjee *(21)* presented data to show that in the presence of inhibitory amounts of cyanide, butyric acid was oxidized to crotonic acid up to a certain point but after this point was reached the oxidation could not continue further because of inhibition of the enzyme by the accumulated crotonic acid. He found that cyanide was a selective inhibitor of the oxidation of butyric acid to ketones by its action on the enzyme system responsible for converting crotonic acid to β-hydroxy butyric acid. The cyanide had no effect on the dehydrogenases which convert butyric acid to crotonic acid. On the basis of this work, Melnick, Luckmann and Gooding *(17)* postulated that sorbic acid inhibited microbial growth and reproduction through the mechanism of inhibition of the dehydrogenase system.

The present investigation was undertaken to determine if sorbic acid is an inhibitor of sulfhydryl enzymes. Evidence is presented to show that sorbic acid is a sulfhydryl enzyme inhibitor, and it is postulated that it may inhibit the growth of microorganisms by inhibition of the myriad sulfhydryl enzymes present in the living cell.

EXPERIMENTAL

Enzymes. The ficin used was a commercial preparation obtained from Merck. The ficin was dissolved in water, let stand at 0°C. overnight and then centrifuged at 70,000 x g. and 0° C. to remove any insoluble material that might be present. Crystalline alcohol dehydrogenase from yeast and crystalline aldolase from rabbit muscle obtained from Worthington Biochemical Corporation were used. The urease, obtained from Nutritional Biochemicals Corporation, was not a crystalline preparation.

Inhibitors. Sorbic acid, obtained from Union Carbide Chemicals Company, was recrystallized 3 times from water before use. The irradiated sorbic acid was prepared by radiating a saturated solution of sorbic acid at 45° C. for 12 hours with an ultraviolet light (Mineralite, Ultraviolet Prod., Inc., South Pasadena, California) using a wavelength of 2540Å. The concentration of sorbic and irradiated sorbic acid was determined by the method of Melnick and Luckmann *(16)*. The maleic acid was prepared from a tech-

nical grade preparation (Nutritional Biochemicals Corporation) by decolorizing with charcoal and recrystallizing 3 times from a water solution.

Inhibition. It has been shown that the inhibition of a sulfhydryl enzyme by maleic acid is a time reaction *(10, 12, 20)*. Figure 1 shows that this is true of sorbic and irradiated sorbic acids also. Therefore, the enzyme was allowed to react for one hour at room temperature (24° C.) with the inhibitors before the activity was determined.

Determination of enzymatic activity. The milk-clotting activity of ficin was determined by the method of Balls and Hoover *(1)* modified as described in a previous publication *(30)*. The esterase activity of ficin was measured by the potentiometric method of Schwert, Neurath, Kaufman and Snoke *(24)* modified to fit the requirements of ficin. The reaction was carried out at pH 6.50 and 35.0° C. using 6.7×10^{-4} molar phosphate buffer and 1.7×10^{-2} molar *p*-toluene sulfonyl-L-arginine methyl ester HCl in a total of 3.0 ml. reaction mixture.

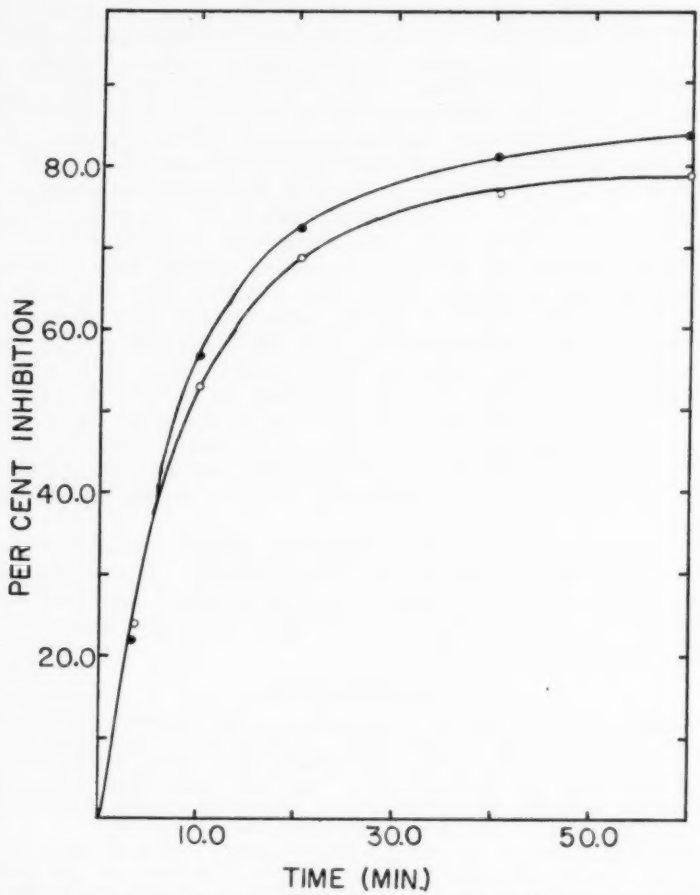

Figure 1. The rate of inhibition of the milk-clotting activity of ficin by sorbic and irradiated sorbic acids. Ficin, at a concentraton of 1.21 mg. per ml. was reacted with 2.82×10^{-3} M sorbic acid (0) and 1.41×10^{-3} M irradiated sorbic acid (●).

Alcohol dehydrogenase activity was determined by the method of Racker *(22)*. Aldolase activity was determined by the method of Sibley and Lehninger *(25)* modified according to Swenson and Boyer *(27)*. The enzyme was allowed to react with the substrate for 10 minutes rather than 30 minutes and the reaction was carried out at 35.0° C. rather than 38.0° C. The urease activity was determined by the method of Sumner *(26)*.

RESULTS

The inhibition of ficin activity with sorbic and maleic acids. Table 1 shows that maleic acid, sorbic acid and irradiated sorbic acid inhibited both the milk-clotting and the esterase activities of ficin under the conditions used here. Krishnamurti and Subrahmanyan *(14)* have also found that maleic acid inhibits ficin activity. Sorbic acid and irradiated sorbic acid were found to be approximately 51 and 83 times more effective, respectively, than the same amount of maleic acid for the inhibition of the milk-clotting activity of commercial ficin.

Sorbic acid and irradiated sorbic acid were found to be equally effective and about 20 times more effective than maleic acid in inhibiting the esterase activity of ficin (Table 1). It should be noted that the inhibitors were present in a concentration 6 times greater in this experiment as compared with the experiments on the milk-clotting activity during the reaction of enzyme and substrate. The effect of dilution and of substrate concentration on the enzyme-inhibitor complex formed after the inhibition period has not been investigated. Table 1 shows that cysteine did not reverse the inhibition under the conditions used here. It is surprising that the irradiated sorbic acid is not more effective than the sorbic acid in reducing the esterase activity of ficin since it is about twice as effective in reducing the milk-clotting activity.

The inhibition of alcohol dehydrogenase activity. Table 2 shows that the activity of alcohol dehydrogenase is inhibited by these acids. Irradiated sorbic acid was found to be only slightly more effective than sorbic acid and these were found to be only two to three times more effective than maleic acid. While maleic acid was just as effective an inhibitor of both ficin and alcohol dehydrogenase, sorbic acid and irradiated sorbic acid were only one-fourth to one-sixth as effective against alcohol dehydrogenase as against ficin (compare Tables 1 and 2). The reason for this is not known. However, one should take note that the alcohol dehydrogenase and milk-clotting assays are carried out at pH 8.5 and 5.2, respectively. The difference in pH may well produce a greater effect on the dissociation constant of the sorbic acid-enzyme complex than it does on the maleic acid-enzyme complex. This remains to be shown experimentally.

It was found that the addition of cysteine, added one hour after the inhibitor and allowed to react for 15 minutes before the assay for activity, did not reactivate the maleic acid-inhibited enzyme; in fact, further activity was lost. But the cysteine did partially reactivate the sorbic acid- and irradiated sorbic acid-inhibited enzyme in contrast to its action on inhibited ficin. The sorbic acid-inhibited enzyme was found to be reactivated more than the irradiated sorbic acid-inhibited enzyme.

The inhibition of aldolase and urease activity. These enzymes were not inhibited to a great extent by the concentrations of inhibitors used (Table 2). Aldolase activity appeared to be slightly inhibited at the higher concentration of sorbic and irradiated sorbic acids but the urease activity was not affected. It has been found by Tsen *(29)* that aldolase is much more resistant to inhibition by sodium selenite, cupric chloride and *p*-chloromercuribenzoate than is alcohol dehydrogenase. Fischer *(7)* reported that urease is inhibited by many of the same compounds, such as allyl isothiocyanate, chloropicrin, iodoacetamide, chloroacetophenone, chloroacetone, β, β-diethyl sulfone and bromopicrin, which inhibit papain activity but that much larger concentrations of these materials must be used. Thus, the sluggish nature of the sulfhydryl group apparently involved in the active centers of aldolase and urease would explain why these enzymes were not inhibited by maleic and sorbic acids in this experiment. Perhaps higher concentrations of the inhibitors would produce an appreciable inhibition of these sulfhydryl enzymes also.

DISCUSSION

Maleic acid has been shown to be an inhibitor of a number of sulfhydryl enzymes such as malic dehydrogenase *(23)*, glutamic dehydrogenase *(13)*, succinic dehydrogenase *(20, 28)*, ficin *(14)*, papain *(10, 20)* glyoxalase *(20)*

TABLE 1

Inhibition of the milk-clotting and esterase activities of ficin by sorbic and maleic acids.
(Reacted for one hour at room temperature (24° C.) and pH 5.0 with inhibitor.)

Maleic acid			Sorbic and irradiated sorbic acids				
				Activity lost (per cent)			
				Milk-clotting		Esterase	
Inhibitor concentration [1] (millimolar)	Activity lost (per cent)		Inhibitor concentration [1] (millimolar)	Sorbic acid	Irradiated sorbic acid	Sorbic acid	Irradiated sorbic acid
	Milk-clotting	Esterase					
1.47	7.05	11.8	0.0294	− 1.12	8.99	—	—
2.94	12.8	18.8	0.0735	3.85	12.5	14.1	17.4
5.88	19.1	31.9	0.147	15.6	29.2	17.4	22.5
8.82	—	35.7	0.294	30.8	49.8	31.4	36.1
11.8	30.0	—	0.588	—	—	45.1	50.2
11.8 + 12.5 millimolar cysteine	33.6	—	0.294 + 12.5 millimolar cysteine	31.4	44.2	—	—
Calculated I_{50} (molar)	2.5×10^{-2}	1.3×10^{-2}		4.9×10^{-4}	3.0×10^{-4}	6.0×10^{-4}	5.9×10^{-4}

[1] Concentration of inhibitor during reaction of inhibitor and enzyme and also during the reaction of enzyme and substrate in the determination of esterase activity. It was 0.17 of this in the presence of substrate during the milk-clotting activity determination.

TABLE 2

Inhibition of alcohol dehydrogenase, aldolase and urease by sorbic and maleic acids.

(Reacted for one hour at room temperature (24° C.) and pH 7.0 with the inhibitor.)

Enzyme	Inhibitor concentration [1] (millimolar)	Activity lost (per cent)		
		Maleic acid	Sorbic acid	Irradiated sorbic acid
Alcohol Dehydrogenase (ADH)	1.47	7.81	31.2	26.0
ADH	2.94	13.0	28.6	33.9
ADH	5.88	20.8	47.4	52.6
ADH	8.82	28.6	60.5	68.3
ADH	13.2	44.8	—	—
ADH	8.82 + 12.5 millimolar cysteine	36.8	10.9	42.5
Aldolase	8.82	− 1.24	1.24	1.65
Aldolase	13.2	− 1.24	5.76	7.00
Urease	8.82	− 0.68	0.34	2.04
Urease	11.8	2.04	− 0.68	− 0.34
ADH	Calculated I_{50} (molar)	1.5×10^{-2}	6.5×10^{-3}	5.5×10^{-3}

[1] Concentration of inhibitor during reaction of inhibitor with enzyme. It was 0.17, 0.22 and 0.50 of this value for alcohol dehydrogenase, aldolase and urease, respectively, during the reaction with the substrate.

and to inhibit the growth and reproduction of cells *(8, 9, 15)*. Morgan and Friedmann *(19, 20)* have shown that maleic acid and sulfhydryl compounds react to form stable compounds. They isolated the addition compounds with thioacetic acid, cysteine and glutathione and showed that they reacted with maleic acid to form *a*-thiosuccinic acid derivatives, $RSCH\text{-}(CO_2H)\text{-}CH_2\text{-}CO_2H$. While maleic acid, the *cis* isomer, is an efficient blocking agent of the sulfhydryl group, fumaric acid, the *trans* isomer, has not been found to be reactive against this group.

On the basis of the similarity in the structure of sorbic acid, $CH_3\text{-}CH = CH\text{-}CH = CH\text{-}COOH$, and maleic acid, $HOOC\text{-}CH = CH\text{-}COOH$, and the similarity in their action on ficin, alcohol dehydrogenase, aldolase and urease it is suggested that sorbic acid inhibits these enzymes by the same mechanism as does maleic acid; i.e. it forms a thiohexenoic acid derivative, $CH_3\text{-}CH = CH\text{-}RSCH\text{-}CH_2CO_2H$. Because of the two double bonds in sorbic acid it can exist in 4 isomeric forms: *trans, trans; trans, cis; cis, trans;* and *cis, cis.* In order for the sorbic acid to react as postulated above it would have to be the *cis, cis* or *cis, trans* isomer. It is thought that the commercial sorbic acid is largely of the *trans, trans* configuration since this would be the most stable form. On the basis of this assumption and the fact that fumaric acid can be converted to the *cis* isomer, maleic acid, by ultraviolet irradiation *(6)* the sorbic acid was irradiated as described in the experimental section. The irradiated sorbic acid was found to be a better inhibitor of ficin than was sorbic acid. The final proof of the postulated mechanism of inhibition of sulfhydryl enzymes by sorbic acid, however, must await the synthesis of the *cis, trans* isomer and its reaction with thioacetic acid, cysteine or glutathione.

The inhibition of the sulfhydryl enzyme systems of microorganisms could certainly explain why many of these organisms cannot grow and reproduce

in the presence of sorbic acid. Some of the important sulfhydryl enzymes found in microorganisms are hexokinase, galactokinase, 6-phosphogluconic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, carboxylase, coenzyme A-linked pyruvic acid oxidase, alcohol dehydrogenase, TPN-alcohol dehydrogenase, potassium-activated yeast aldehyde dehydrogenase, TNP-linked aldehyde dehydrogenase, aldehyde dehydrogenase, butyryl coenzyme A dehydrogenase, acetate kinase, succinic dehydrogenase, amino acid reductases, histidase, tryptophanase, aspartase, DPNH cytochrome C reductase and ribonuclease. This is not a complete list and differences would undoubtedly be found in the rates of reaction of these enzymes with sorbic acid. For an excellent review on the thiol groups of biological importance the reader is referred to the work of Barron *(2)*. In view of the many sulfhydryl enzymes in microorganisms and the demonstrated effectiveness of sorbic acid in inhibiting these enzymes it is not surprising that their growth is inhibited. What is more surprising is that certain bacteria can grow quite well on media containing sorbic acid *(4, 31)*. Apparently, these organisms have a mechanism for metabolizing or detoxifying the sorbic acid.

SUMMARY

Sorbic acid has been shown to be an inhibitor of the sulfhydryl enzymes ficin and alcohol dehydrogenase at concentrations of 10^{-4} molar and is more efficient than maleic acid. Aldolase and urease were not significantly inhibited by sorbic and maleic acids under the conditions used in this work. Irradiated sorbic acid was found to be a better inhibitor of ficin than sorbic acid.

It is suggested that the mechanism of inhibition of the sulfhydryl enzymes by sorbic acid and similar α, β-unsaturated acids is similar to the one found for maleic acid. It is further postulated that this is the mode of inhibition of the growth and reproduction of microorganisms.

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DESTRUCTION OF *CLOSTRIDIUM BOTULINUM* BY IONIZING RADIATION

I. IN NEUTRAL PHOSPHATE AT ROOM AND FREEZING TEMPERATURES[a, b]

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(Manuscript received May 31, 1958)

In the radiation sterilization of food, *Clostridium botulinum* assumes major importance since it appears to be the most radiation resistant not only of the spore-forming food spoilage organisms but also of the food poisoning organisms. An extremely radio-resistant micrococcus *(2)* has been reported; yet it is apparent that any non-sporing organism will be destroyed by the heat treatment necessary to inactivate the enzymes in the foods to be preserved. Morgan and Reed *(11)* have previously reported that spores of *Cl. botulinum* appeared more resistant to radiation than spores of several other food spoilage organisms. A knowledge of requirements for destruction of *Cl. botulinum* is essential for the development of preservation procedures which are safe from the health standpoint.

The Research Laboratories of the National Canners Association, the American Can Company, and the Continental Can Company have joined in a cooperative program to determine the radiation dosage needed to produce commercial or absolute sterility in five different canned foods inoculated with a mixture of strains of *Cl. botulinum.* The program includes direct incubation of the irradiated products to determine the spoilage end-points and subculture counts on both the products and the phosphate solution. The subculture counts are for the purpose of constructing irradiation survival curves in neutral phosphate buffer solution and in each food, and determining whether viable cells or spores remain in products showing no evidence of spoilage. This paper discusses results of spore recovery tests on irradiated phosphate solution and compares the survival curves with those obtained in irradiated foods.

It is well known to those interested in the heat resistance of micro-organisms that a standard reference medium is of aid in comparative studies. In 1922 Esty and Meyer *(6)* introduced the use of phosphate buffer solutions in studies on the heat resistance of *Cl. botulinum.* They found that M/15 neutral Sorenson buffer solution provided an easily reproducible reference medium which changed little, if any, during prolonged heating. This proved

[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, May 27, 1958.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number *891* in the series of papers approved for publication.

to be so useful that the resistance in neutral phosphate solution is now commonly called the "normal" heat resistance. The "classic" heat resistance of *Cl. botulinum (6)* was determined in this heating medium, and the thermal death time curve has been corrected for lag time by Townsend, Esty and Baselt *(14)*. The latter authors discussed the practice of heating spores in standard phosphate and in food to determine the ratio of resistance in food over resistance in phosphate. They pointed out that such a food:phosphate factor could be applied to different spore suspensions only if the slope (z value) of their thermal death time curves were identical.

Phosphate buffer solution was used as a standard substrate in the present radiation resistance studies with the hope that a food:phosphate factor might be developed during future comparative investigations.

EXPERIMENTAL PROCEDURE

Spore suspensions. In preparing the test spore suspensions, the following strains of *Cl. botulinum* were used: Type A — No. 62, 73, 78 (from K. F. Meyer), No. 12885 and 13824 (from NCA, Berkeley); Type B — No. 32, 113, 213 (from K. F. Meyer), No. 12033 and 13983 (from NCA, Berkeley). Each of these strains was grown separately in Wheaton's Beef Heart-Casein Broth *(15)* by incubating 17 days at 30° C. (86° F.) followed by 10 days at room temperature. They were then filtered through cheesecloth and centrifuged. All the supernatant liquid was poured off and the spores were resuspended in sterile tap water. They were heated for 10 minutes at 170° F. to inactivate vegetative cells and toxin, and colony counts were made in Anderson's medium *(1)* containing sodium bicarbonate. The strains were then mixed to produce one suspension containing the spores of the 10 strains in equal numbers.

Phosphate studies. Both M/15 and M/60 phosphate buffers were examined for suitability in these tests because of the toxicity of buffers during heating reported by Williams and Hennessee *(16)*. Preliminary tests with this spore suspension showed that freezing in M/15 Sorenson phosphate buffer allowed a greater spore recovery than freezing in M/60 phosphate buffer. Consequently, the M/15 buffer at pH 7.0 was used in these tests. It was prepared as follows: Na_2HPO_4 — 5.675 g. and KH_2PO_4 — 3.63 g. per liter distilled water. Sterilization was under steam pressure at 121° C. (250° F.) for 30 minutes.

The spore suspension was added to the sterilized neutral buffer solution so that each ml. contained approximately 100,000 spores. One milliliter of the inoculated buffer solution was added to each sterile pyrex glass tube of 7 mm. inside diameter and 9 mm. outside diameter. Tubes were heat sealed at a length of 2⅜ inches so that they would fit inside 202 x 214 cans.

Tubes were frozen at − 20° C. (− 4° F.). Six tubes were placed in the center of a can partially filled with cold solidified agar, at equal distances from the sides of the can yet not touching each other. Warm agar was then poured over the tops of the tubes and rapidly cooled. Cans were then sealed and held at freezing temperature.

The following day the tubes, in the cans, were radiated at Argonne National Laboratories by gamma radiation from the mixture of fission products in spent fuel elements. The cans were cycled as necessary in the urns and irradiation racks to give uniform dosage and temperature control. Tubes radiated in the frozen condition received an average rate of 20,740 rad per minute and those radiated at room temperature received an average rate of 27,810 rad per minute. All tubes were stored at about −20° C. until subcultured for recovery counts.

Phosphate tubes were aseptically opened, emptied into dilution blanks or media tubes, and rinsed twice with the diluent. Triplicate dilution counts were made at dosages up to 927,000 rad, and tubes irradiated to this and higher dosages were subcultured directly into the recovery medium. Incubation was at 30° C. (86° F.) and the counts were determined in 3 days. Excessive gassing occurred during longer incubation.

The medium used for counting was NCA liver broth *(12)* substituting tryptone for peptone and adding 0.1% soluble starch, 0.05% sodium thioglycollate, and 1.5% agar. The medium was tubed in 20 ml. amounts in modified deep agar shake tubes *(9)* and

sterilized under steam pressure at 121° C. (250° F.) for 30 minutes. Two tenths ml. of filter sterilized sodium bicarbonate was added to each tube of medium after cooling to 55° C. (131° F.) and immediately before culturing.

Food studies. Blanched fresh peas, frozen cream of chicken soup, and ground parboiled pork were inoculated with spores of the same mixed suspension described above to give 10,000 spores per gram in 202 x 202 cans holding approximately 100 g. each. The cans were held at − 20° C. (− 4° F.) until irradiated. Radiation procedures were similar to those listed above except that the foods were radiated only under frozen conditions. The average dose rate was 24,400 rad per minute.

After radiation, the cans for spore recovery tests were stored at about − 20° C. and held until subcultured. Immediately before opening and culturing they were thawed by immersion in warm water.

The cans were aseptically opened and the contents blended in a sterile commercial blender. A 5-tube series most probable number count was made on each of 6 cans of food which had received a given radiation dose. In most instances, a total of 55.5 ml. of the can contents were cultured, with 8 radiation doses plus unirradiated controls being used. Inoculated tubes were heated in a water bath for 5 minutes at 77° C. (170° F.) to destroy vegetative contaminants, partially cooled, stratified with sterile vaseline, and incubated at 30° C. (86° F.) for 4 weeks. Growth was indicated first by turbidity and next by the vaseline plug rising due to gas production.

The medium for sub-culture-counting of the viable spores in the irradiated products was the liver broth previously described, used without agar and with the same proportion of sodium bicarbonate.

Toxicity tests. Toxin was determined by subculturing in liver broth a colony from a positive tube from each radiation dose where survivors were recovered in the phosphate experiment. The liquid from the positive product subculture tubes at each radiation dose was used. At least 4 days after growth appeared, 0.2 ml. of the growth liquid filtered through sterile cotton was injected intraperitoneally into a Webster Swiss white mouse of about 20 g. weight. If the mouse died, 0.4 ml. of a mixture of equal parts of bivalent (A & B) anti-toxin and growth liquid was injected into a second mouse. If this second mouse lived, toxin was presumed confirmed.

RESULTS AND DISCUSSION

One part of this investigation was to determine the survivor curve of the *Cl. botulinum* suspension in neutral phosphate. Two irradiation temperatures were used for purposes of comparison. Biological control tests showed no significant spore germination during the radiation period. The results when plotting the logarithm of the percent survivors indicate that this mixed Type A and Type B suspension was more resistant to irradiation at freezing than at room temperature. Kempe *et al. (8)* have also found that *Cl. botulinum* spores have differing destruction rates when irradiated at different temperatures in neutral phosphate. At the two irradiation temperatures used in the present study the effects upon spore destruction rates were comparable to those reported by Kempe. To determine graphically whether the indicated difference due to temperature during radiation was more apparent than real, the highest and lowest counts of the 6 tubes cultured from each radiation dose were used to plot areas of survival (Figure 1). The fact that there is no overlapping of the two survival areas tends to confirm the reality of a temperature effect. Statistically it was found that the difference between survival at the two radiation temperatures was significant at the 95% confidence limits for each dosage except 810,000 rads (Table 1). At the present time it is not known whether there would be a similar effect on survival in foods irradiated at the two temperatures. Denny, Bohrer, and Reed *(5)* reported a comparable but opposite effect of temperature when spores of *Cl. botulinum* No. 62-A were radiated in peas.

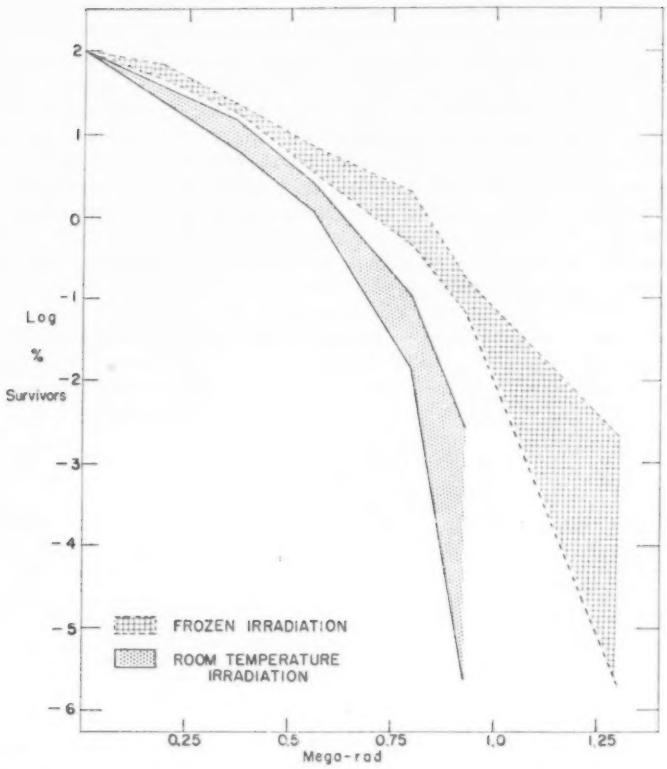

Figure 1. Areas of survival based on spore counts of Clostridium botulinum in neutral phosphate buffer irradiated at frozen and room temperature.

TABLE 1

Comparison of Clostridium botulinum spore counts following irradiation at room and frozen temperatures in neutral phosphate

Radiation dose	Actual range of spore counts				95% Confidence limits			
	Room		Frozen		Room		Frozen	
(rad)								
0	60,000 -	80,000	70,000 -	100,000	57,000 -	85,000	61,000 -	111,000
370,000	4,400 -	10,500	16,000 -	25,000	4,800 -	13,000	13,000 -	27,000
570,000	800 -	1,800	3,100 -	5,200	700 -	2,100	2,300 -	6,100
810,000	10 -	230	400 -	1,700	0 -	230	65 -	1,800
930,000	<0.17 -	2	48 -	157	0 -	2.6	4 -	190
1,300,000	<0.17 -	<0.17	<0.17 -	2	0		0 -	2.2
1,800,000	<0.17 -	<0.17	<0.17 -	<0.17	0		0	

Spores of *Cl. botulinum* in food have previously been shown to be more resistant to radiation than the same spores in water *(5)*. The protective effect of food is demonstrated also by results shown in Figure 2 comparing survival in neutral phosphate buffer solution with survival in peas and pork during radiation under frozen conditions. Although not shown, results on cream of chicken soup are comparable to those shown for peas in this figure. The radiation resistance of *Cl. botulinum* under frozen conditions was greatest in the ground par-boiled pork, less in peas and least in neutral phosphate buffer solution. The nature of the factors responsible for the differences is not known. From these results, it might appear that density alone might account for the difference. However, previous work has shown that density is not the only factor involved *(5)*.

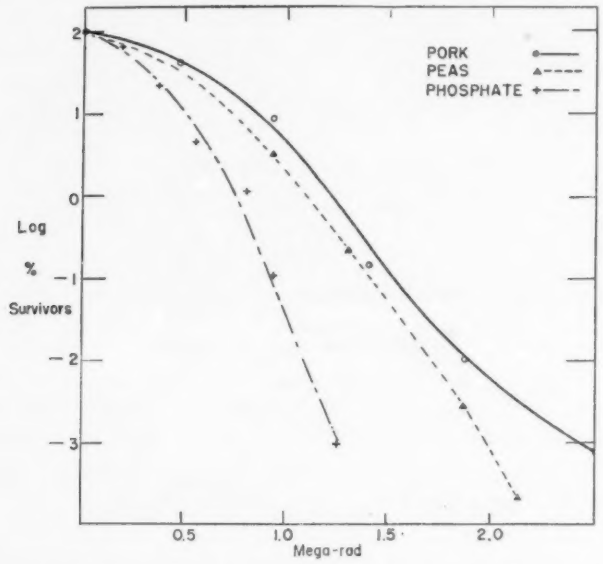

Figure 2. Survivor curves for spores of Clostridium botulinum in different substrates irradiated in the frozen state.

These survivor curves could not be accurately defined by a single straight line through the origin (Figure 2). However, a single straight line through the origin and a point representing the limit of recoverable survivors may be drawn and used for comparative purposes. The slope of this survivor curve (number of rads required to traverse one log cycle) represents a tenfold decrease in number of spores surviving and may be called the radiation D value or the radiation decimal reduction dose. It is thought that this is comparable to the thermal decimal reduction time of Katzin *et al. (7)*. In heat resistant studies, the end points representing survival and destruction at different temperatures may be plotted against time to give a thermal death time curve. The slope of the thermal death time curve, or z (in °F.) can then be used in

calculating heat processes *(3, 4)*. This provides a method of verifying experimental results since any number of different temperatures may be used as a variable to define the thermal death time curve. However, in the case of radiation one necessarily determines the radiation death dose rather than radiation death time. The effect of a given dose (in rads) is apparently the same whether the dose is received in minutes or hours *(10, 13)* and whether received from gamma or cathode rays *(13)*. Therefore, the sterilizing dose or radiation process might be determined directly from the radiation survivor curve D values. However, our tests with varying initial concentrations (Table 2) have shown apparent increases in D value as the initial spore concentration is increased. In view of this and the non-lineality of survivor curves, radiation sterilization requirements should be determined from complete survivor curves for the maximum initial spore concentration rather than using survival data (D values) obtained with lower spore concentrations. The radiation sterilization dose for foods should be verified by relatively large-scale inoculated packs to ensure safety from the public health standpoint.

TABLE 2

Variations obtained in radiation decimal reduction doses of Clostridium botulinum (irradiated at room temperature)

Type	Radiation source	Initial spore concentration in phosphate	D value
		(per ml.)	*(from survivor curves)*
5 Strains	Spent fuel rods (MTR)	46,000	180,000 rad
each A and B	Spent fuel rods (Argonne)	76,000	230,000 rad
Strain No.	Cobalt 60 (Brookhaven)	4,200,000	350,000 rad
62 - A	Cobalt 60 (Brookhaven)	82,000,000	380,000 rad

SUMMARY

Neutral phosphate buffer was used as a standard reference medium for cultures subjected to irradiation. The relationship between irradiation in the reference medium at freezing and room temperatures was discussed. *Cl. botulinum* was shown to be more resistant to radiation in foods than in neutral phosphate when irradiated at freezing temperatures. The use of data obtained from radiation survivor curves was discussed.

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DESTRUCTION OF *CLOSTRIDIUM BOTULINUM* BY IONIZING RADIATION
PART II. PEAS, CHICKEN SOUP, AND PORK IN THE FROZEN STATE [a, b]

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(Manuscript received May 31, 1958)

Determination of the radiation dose required to destroy spores of the toxin producing organism, *Cl. botulinum*, and produce a safe, sterile product is essential before irradiated foods can be introduced to consumers. The magnitude of cost and of such problems as changes in flavor, odor, texture, and nutrients, of course, depends very largely upon the radiation dose required for sterilization.

Six-hundred cans each of peas, cream of chicken soup, and parboiled pork were inoculated with approximately 1,000,000 spores of *Cl. botulinum* per can and subjected, in the frozen state, to various levels of gamma radiation from mixed fission products in spent fuel elements.

Counts were made of surviving organisms in representative samples after irradiation and the remaining samples placed in incubation at 86° F. (30° C.) for one year.

Counts of surviving spores, as well as tests for botulinum toxin, were made on flat cans surviving the year of incubation.

EXPERIMENTAL PROCEDURE

Inoculum. Spore suspensions used throughout consisted of a mixture of 5 strains of *Cl. botulinum*, Type A, and a mixture of 5 strains of Type B, all of which were grown separately on Wheaton's beef heart casein medium *(7)*, concentrated, suspended in water, and heat shocked 10 minutes at 170° F. (77° C.) The 2 mixtures were provided by C. T. Townsend of the Western Laboratory of the National Canners Association. Dilutions of the original suspensions were made to permit inoculation of approximately 5,000 spores of each type per gram of product.

Peas. Peas for this pack, Perfection variety garden run, were blanched 3 minutes in boiling water, cooled, and frozen until ready for use. Sixty-one grams of peas were weighed into each 202 x 202 can and the mixed spore inoculum added by pipette. Cans were brined with 36 ml. cold brine (1.4% sodium chloride, 4.8% sucrose) and closed at 58-65°F. (14-18°C.) under 25 inches of mechanical vacuum.

Chicken soup. Concentrated cream of chicken soup was removed from a commercial line without thermal processing and was returned frozen to Barrington for inoculation. Ninety-eight grams were filled into each 202 x 202 can and mixed spore inoculum added by pipette. Contents were stirred to insure distribution of spores, and the cans were closed at 55-58°F. (13-14°C.) under 25 inches of mechanical vacuum.

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[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 27, 1958.

[b] This paper reports research undertaken in co-operation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned Number 903 in the series of papers approved for publication.

Pork. Cellar butts (boned pork shoulder) were cut into strips, ground through a ½-inch screen, and 260 pounds parboiled in 3 batches in a 40-gallon steam jacketed kettle with 24 gallons of water. After simmering for 30 minutes, each batch of meat was removed and chilled. The liquor was retained for the next batch of meat. The shrinkage on parboiling was approximately 41%.

The parboiled pork (133 lbs.) was ground through a ⅛-inch screen after chilling overnight and mixed for eight minutes under 25 inches of vacuum with liquor from parboiling (13.3 lbs.) which had been inoculated with a mixture of spores of *Cl. botulinum* calculated to yield 10,000 spores per gram of canned product.

Irradiation. All irradiation and dosimetry was performed at Argonne National Laboratory under supervision of Miss H. Gladys Swope*. The mixture of fission products in spent fuel elements served as the source of gamma radiation. Fuel elements are arranged in an underwater rack in such a way that samples for irradiation may be placed between the elements in cylindrical urns. During radiation the urns are rotated to insure symmetrical distribution of radiation dose about the axis.

Ten 202 x 202 cans at a time are placed in specially constructed polystyrene foam insulating cylinders with ¾-inch thick walls. This cylinder, containing 10 cans, fits into the aluminum urn which is immersed and placed between fuel elements for radiation (Figure 1).

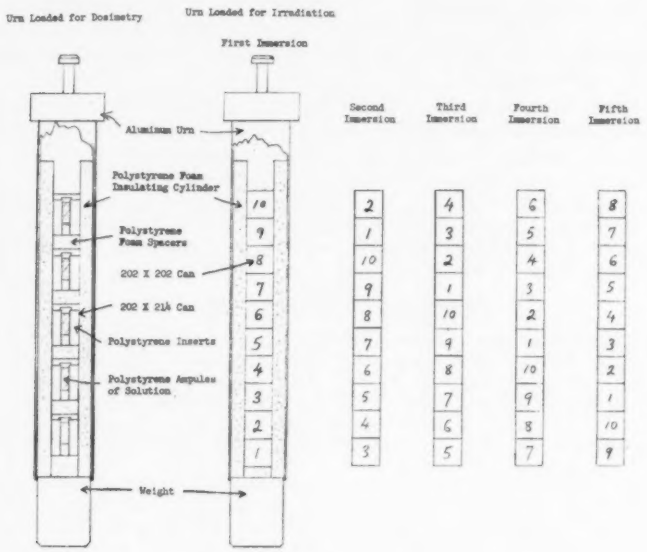
Urn Loaded for Dosimetry
Urn Loaded for Irradiation
First Immersion
Second Immersion
Third Immersion
Fourth Immersion
Fifth Immersion
Aluminum Urn
Polystyrene Foam Insulating Cylinder
Polystyrene Foam Spacers
202 X 202 Can
202 X 214 Can
Polystyrene Inserts
Polystyrene Ampules of Solution
Weight
10 9 8 7 6 5 4 3 2 1
2 1 10 9 8 7 6 5 4 3
4 3 2 1 10 9 8 7 6 5
6 5 4 3 2 1 10 9 8 7
8 7 6 5 4 3 2 1 10 9

Fig. 1

Cans were immersed 5 times, each time in a different order, permitting a substantially equal dose for all cans in spite of variations in gamma flux along the axis of the cylinder. Between immersions in the radiation source, samples were removed and chilled in dry ice-alcohol to maintain the product in the frozen state throughout the radiation period. On 868 internal temperature readings, cans started the radiation period at an average of − 19° F. (− 28° C.) and finished at an average of − 1° F. (− 18° C.), with individual variations.

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* Group Leader, Chem. Engineering Division.

Dosimetry. Radiation dose rate determines the time of immersion required for a given dosage. This rate was determined daily by preliminary irradiations of ferrous-cupric dosimeter solution composed of .01 N H_2SO_4, .001 M $FeSO_4$, and .001 M $CuSO_4$ in distilled water. The amount of ferric ion produced after an accurately measured exposure of approximately 20 minutes was analyzed by Beckman D. U. spectrophotometer at 3200 A, and the dose rate was calculated using a G-value of 0.66 molecules/100 e.v. *(3)*.

Incubation. With the exception of 6 cans of each food product irradiated at each dose level, all cans were incubated at 86° F. (30° C.) for one year.

Survivor counts. Six unincubated cans of peas and chicken soup at each radiation level were opened aseptically and surviving spores counted using most probable number techniques *(1)*.

After a year of incubation at 86°F. (30°C.) from 6 to 25 flat cans of peas and chicken soup from each irradiation level were subcultured for evidence of viable spores. Where evidence of viable organisms was obtained, subcultures were tested for toxin by intraperitoneal injection into mice with and without antitoxin *(1)*.

Cans were washed in a chlorine solution and the end to be opened was flamed and a hole one inch in diameter was cut with a bacteriological opener *(6)*. Contents were added to an Oster Frapaizer and blended under vacuum. The blended product was then cultured into freshly exhausted liver infusion to which had been added .1 ml. of 10% Seitz filtered sodium bicarbonate for tubes of 10 ml. media, and .4 ml. for tubes of 40 ml. aliquots of media. Five tubes each were inoculated with 10 ml. of blended product, 5 with 1 ml. portions and 5 with .1 ml. aliquots. After inoculation all tubes were heated 5 minutes at 170°F. (77°C.) then partically cooled and stratified with sterile vaseline. All inoculated tubes were incubated 4 weeks at 86°F. (30°C.).

The medium was prepared from fresh beef liver infusion to which was added 10 g. Tryptone, 1 g. soluble starch, 1 g. K_2HPO_4, and .5 g. of sodium thioglycollate. The pH was adjusted to 7.1-7.2 with 10% sodium hydroxide.

Toxicity tests. All swelled cans of each product, up to a maximum of ten per dose level, were tested for toxin. Brine from peas (.2 ml.) was injected into a mouse intraperitoneally. If the mouse died, an additional .2 ml. brine was mixed with an equal volume of polyvalent antitoxin (Types A, B and C), and the mixture was injected into a second animal. The concentrated chicken soup was first centrifuged and .2 ml. of supernatant liquid injected as above. The pork was diluted with an equal volume of sterile distilled water, mixed in a Waring blender and .2 ml. supernatant liquid injected.

All flat cans of peas and chicken soup were tested as described above, except that composite samples were prepared from contents of 8 to 10 cans. Samples of chicken soup to be composited were mixed in an Oster Frapaizer prior to centrifugation. Samples of each individual can were retained in the event a composite sample should prove toxic.

RESULTS AND DISCUSSION

Since chemical dosimetry was customarily performed in replicate, an excellent opportunity was provided for measuring the reproducibility of the irradiation dose. Standard deviations of 1,220 paired analyses of dose rate were subjected to statistical analysis. Standard deviation of duplicate readings was determined to be 790 rep/min. Since each radiation time was based on multiple determinations (8.8 on the average), the standard error in the radiation time is about:

$$\frac{790}{\sqrt{8.8}} = 266 \text{ rep/min.}$$

The average dose rate from 1,447 determinations was 2.62×10^4 rep/min., so that the coefficient of variation is about:

$$\frac{266}{26{,}200} = 1\%$$

Thus, even if we demand 99% confidence limits, the recorded dose should lie within ± 2.5% of the true dose as defined by the dosimetry procedure.

Radiation doses are reported in terms of rep_{93} which is equivalent to 0.93 rad. For example, 3 million rep_{93} equal 2.79 million rad.

Survivor counts of spores irradiated in peas, chicken soup, and pork are presented in Tables 1 through 3. The general multi-hit curve *(5)* was fitted using the objective statistical procedure of Kimball *(4)* as modified by Gurian *(2)*. Unfortunately, this method, which has proven useful with relatively non-resistant organisms, fits experimental points at the top of the curve very closely at the expense of points several log cycles down. In spite of some shortcomings in the fit, however, Figure 2 does demonstrate that the familiar humpbacked survival curves are entirely consistent with the theory that more than one hit is required for inactivation of these spores. Perhaps studies designed to that end can offer some information about the interior of the spore. At any rate, the multi-hit curve never becomes concave upwards, a fact which may be reassuring to those concerned with a safe radiation dose for foods.

TABLE 1

Survival of Cl. botulinum irradiated frozen in 202 x 202 cans of peas

Dose	Average count surviving spores per gram in 6 cans	Per cent swell spoilage of 94 cans	Surviving spores per gram in 6 flat cans
	(Unincubated)	(Incubated 1 year) [3]	(Incubated 1 year) [3]
(Mrep)			
0	13,000	100% [1]	
1.0	410		
1.4	28		
2.0	.35	77%	0
2.3	.029	19%	.003 [2]
2.6	0	27%	0
3.0	0	0	0
3.5	0	0	0
4.0	0	0	0

[1] 12 cans.
[2] One can in 6 contained spores.
[3] At 86° F.

TABLE 2

Survival of Cl. botulinum irradiated frozen in 202 x 202 cans of chicken soup

Dose	Average count surviving spores per gram in 6 cans	Per cent swell spoilage of 94 cans	Surviving spores per gram in 6 flat cans
	(Unincubated)	(Incubated 1 year) [5]	(Incubated 1 year) [5]
(Mrep)			
0	24,000	100% [1]	
1.0	2,000		
1.4	51		
2.0	.23	100%	
3.0	.003	4%	0
3.5	0	1%	0
3.6	0	1% [2]	.004 [3]
3.8	0	0	.005 [4]
4.0	0	0	0

[1] 12 cans.
[2] Contents of this can were non-toxic.
[3] Two cans in 24 contained spores.
[4] Three cans in 25 contained spores.
[5] At 86° F.

TABLE 3

Survival of Cl. botulinum irradiated frozen in 202 x 202 cans of pork

Dose	Average count surviving spores per gram in 6 cans (Unincubated)	Per cent swell spoilage of 94 cans (Incubated 6-12 mos.) [4]
(Mrep)		
0	11,800	100% [1]
.5	4,360	
1.0	980	
1.5	16	
2.0	1.3	100%
3.2	0	11% [2]
3.5	0	1%
3.7	0	2% [3]
4.0	.04	0
4.5	0	0

[1] 12 cans.
[2] Four cans of 9 tested were non-toxic.
[3] Both cans were non-toxic. One sour and not putrid; one putrid.
[4] At 86° F.

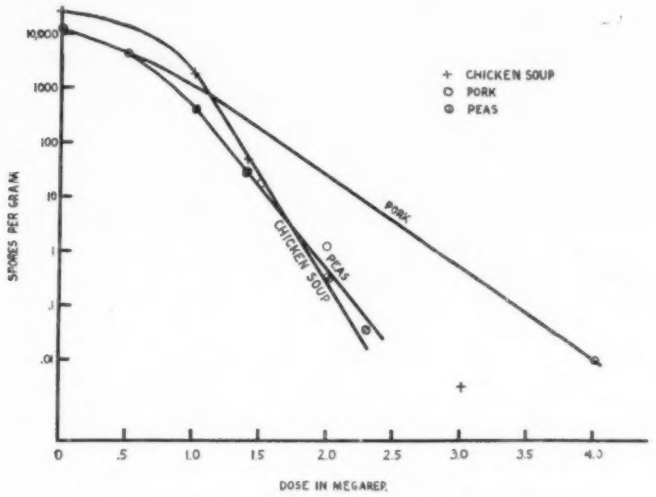

Fig. 2. Survival of Cl. botulinum in irradiated foods.

Percentage of swell spoilage in 94 cans at each dose level after incubation is presented in the same tables. Seven swelled cans which did not contain toxin are indicated in footnotes. Sixty-one other swelled cans tested all produced toxic reactions. No evidence of toxin was discovered in any of the 449 flat cans of peas or 433 flat cans of chicken soup tested.

Viable toxin producing cultures were recovered from one flat can in 36 flat cans of peas subcultured. Viable toxin producing cultures were also recovered from 5 flat cans in 72 cans of chicken soup. Thus, it is apparent that absence of swell spoilage is not fully indicative of the complete destruction of spores.

SUMMARY

Toxic swells developed in peas inoculated with 10,000 spores per gram of *Cl. botulinum* which had received 2.5 Mrep, but no swell spoilage developed after 3.0 Mrep. Toxic swells developed in similarly inoculated chicken soup which had received 3.5 Mrep, and a non-toxic, putrid swell developed even at 3.6 Mrep, but no swell spoilage developed after 3.8 Mrep. Toxic swells developed in inoculated pork after 3.5 Mrep, and non-toxic swells (one putrid) developed at 3.7 Mrep; no swell spoilage developed after 4.0 Mrep.

Viable spores were isolated from occasional flat cans of peas and chicken soup incubated one year at 86° F., but in no case was toxin encountered in any flat cans.

It seems likely that no single dose will be best for all products since resistance of spores is different in different media.

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STUDIES ON PURIFIED TYPE A *CLOSTRIDIUM BOTULINUM* TOXIN SUBJECTED TO ULTRACENTRIFUGATION AND IRRADIATION [a]

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In previous experiments large dosages of Cobalt-60 radiation were required to inactivate crude *Clostridium botulinum* toxin in broth and surface ripened cheese *(5)*. This pattern of inactivation of the toxin suggested that the toxin may exist in more than one form. Wagman and Bateman *(6)* reported the dissociation by ultracentrifugal fractionation of a 1% solution of crystalline type A *Cl. botulinum* toxin. This dissociated toxin was found to be made up of two different molecular components; one a molecular weight of approximately 70,000 and the second, one of 1,000,000.

The primary purpose of this investigation was to study the effect of radiation on the two dissociated components of purified toxin in comparison with the undissociated purified product.

MATERIALS AND METHODS

In each of 3 experiments approximately 15 mg. of purified type A *Cl. botulinum* toxin in 5 ml. of 0.05M sodium acetate-acetic acid buffer at pH 3.8 was placed in a Visking casing and dialyzed against 0.05M sodium phosphate buffer, pH 7.5, rotated for 6 hrs. at 4° C. in a cylinder, and then dialyzed without rotation overnight against a second portion of buffer. Potency of the toxin solution after dialysis was $80 \times 10^6 \pm 20\%$ LD_{50} per ml.

The dissociated toxin was centrifuged for 3 hours in an average field strength of 100,000 times gravity at 0° C. to separate it into 2 components. The supernatant fluid was removed and stored at 4° C. The pellet was dissolved in 0.05M sodium phosphate buffer, pH 7.5, with the aid of a glass stirring rod. The final volume of 10 ml. was stored at 4° C. Each day that radiations were made, dilutions estimated to have 100,000 MLD per ml. were prepared in sodium phosphate buffer from these concentrated solutions and from the solution of whole toxin. Three ml. portions of diluted toxin were pipetted into 15 x 125 mm. pyrex culture tubes. The air was replaced with a mixture of 90% N_2 and 10% CO_2. Tubes were constricted approximately one inch above the surface of the sample and sealed.

One tube of each fraction was radiated with Cobalt-60 at several levels in order to establish an inactivation pattern for the whole toxin and for each of the 2 components separated by ultracentrifugation. Temperature of radiation varied between 3.5° and 8.0° C. After each radiation exposure the tubes were opened, appropriate dilutions were made in gelatin-phosphate diluent *(3)* and 16 to 20 g. white mice were injected intraperitoneally. For the irradiated samples 4 mice were inoculated with each of several dilutions. For each unirradiated sample 3 mice were inoculated with each dilution. Three separate series of inoculations were used to establish the toxicity of the unirradiated specimens. The mice were observed for 4 days.

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RESULTS

Data from irradiation studies on three 5 ml. portions of purified type A *Cl. botulinum* toxin are given in Tables 1, 2 and 3.

There was little difference in the inactivation patterns of whole purified toxin and the 2 fractions (see Table 1). Both fractions, as well as the parent toxin, were reduced to appreciably lower toxicities by 60,000 rads, but subsequent reductions in toxicity occurred at a slower rate. At dosages above 60,000 rads, the bottom fraction agreed more closely with the inactivation pattern of the whole toxin than with the top fraction.

The initial concentration of toxic material, within the limits used in this experiment, did not seem to affect the irradiation dosage necessary for inactivation to the end point (less than 5 MLD per ml.). The initial concentration of the whole toxin was 50,000 MLD, and 100,000 MLD for both top and bottom fractions. The dosage necessary to establish the end point was 730,000 rads for all 3 toxic fractions studied.

The inactivation pattern for each of the 3 toxin solutions tested in Experiment II was similar (see Table 2), indicating that type A *Cl. botulinum* toxin particles of differ-

TABLE 1

Effect of irradiation with Cobalt-60 on purified Type A Clostridium botulinum toxin in 0.05M sodium phosphate buffer solution (pH 7.5)

Irradiation dosage (rads) (000 omitted)	Whole toxin concentration MLD	Supernatant concentration MLD	Pellet concentration MLD
None	50,000	100,000	100,000
3	20,000	20,000	10,000
6	2,000	5,000	5,000
15	500	1,000	1,000
30	200	200	500
60	20	20	100
120	10	20	20
180	10	20	20
270	10	10	20
360	10	10	20
460	5	5	10
550	5	5	10
730	Less than 5	Less than 5	Less than 5

TABLE 2

Effect of irradiation with Cobalt-60 on purified Type A Clostridium botulinum toxin in 0.05M sodium phosphate buffer solution (pH 7.5)

Irradiation dosage (rads) (000 omitted)	Whole toxin concentration MLD	Supernatant concentration MLD	Pellet concentration MLD
None	100,000	100,000	100,000
3	20,000	20,000	20,000
6	20,000	20,000	10,000
15	5,000	5,000	2,000
30	1,000	1,000	1,000
60	200	200	200
120	200	100	100
180	50	50	50
270	20	20	20
360	20	20	20
460	20	20	20
550	20	20	20
730	20	10	10
900	10	10	10
1,100	5	Less than 5	Less than 5
1,500	5	Less than 5	Not done

TABLE 3

Effect of irradiation with Cobalt-60 on purified type A Clostridium botulinum toxin in 0.05M sodium phosphate buffer solution (pH 7.5)

Irradiation dosage (rads) (000 omitted)	Whole toxin concentration MLD	Supernatant concentration MLD	Pellet concentration MLD
None	100,000	50,000	50,000
3	10,000	20,000	20,000
6	5,000	10,000	10,000
15	1,000	5,000	5,000
30	500	200	1,000
60	200	100	500
120	20	50	500
180	20	50	200
260	10	20	100
350	10	10	100
440	5	10	100
530	5	10	50
710	Less than 5	5	20
880	Less than 5	Less than 5	20
1,000	Less than 5	Less than 5	20
1,400			10
1,800			Less than 5

ent molecular weights are affected by gamma irradiation in the same manner (about 80% of the toxicity was removed by only 3,000 rads, whereas it required 900,000 rads to inactivate 99.99% of the toxin).

The initial toxicity measured by mouse titration was between 100,000 and 200,000 MLD for each portion. The relative toxicity was a maximum near 200,000 MLD for the whole toxin and somewhat closer to 100,000 MLD for the supernatant and the diluted pellet. The whole toxin was higher in initial toxicity than either fraction and required more radiation for inactivation than the top and bottom fractions.

In Experiment III the initial concentration of the whole toxin was 100,000 MLD, and 50,000 MLD for both top and bottom fractions (see Table 3). The inactivation patterns were not as uniform as were observed in the other 2 experiments.

The pH level of 0.05M sodium phosphate buffer was not altered by 360,000, 460,000, 550,000, 730,000, 1,100,000 and 1,500,000 rads.

DISCUSSION

Results of this study showed that there was little difference in the inactivation patterns of whole purified type A *Cl. botulinum* toxin and its two dissociated fractions separated by ultracentrifugation.

Approximately 15 ml. of concentrated toxin solution was brought to our laboratory in a tightly stoppered 15 × 150 mm. culture tube. For each of the first 2 experiments approximately one-third of the solution was transferred with a syringe to a Visking casing prior to dialysis. In the last experiment, however, the remainder of the solution was poured from the culture tube into the dialysis bag. A thin film of toxin solution probably clung to the upper wall of this tube long enough during storage to be at least partially denatured and consequently slightly reduced in toxicity. Therefore, it is possible that the inclusion of some of the residual material clinging to the upper wall of the culture tube may have contributed to the results observed in Experiment III when these data are compared to Experiments I and II.

Based on the first two experiments with similar portions of purified type A *Cl. botulinum* toxin solution, it appeared that there was no difference in the

gamma irradiation sensitivity of the whole toxin and its two dissociated fractions. The data from the third experiment were not as uniform. The inactivation pattern for the supernatant agrees more nearly with that of two earlier experiments. The fact that the diluted pellet exhibited greatest resistance to inactivation suggested that possibly the initial toxicity of the heavier particles was low because of a slow degradation in storage, and that the greater amount of solid material present was giving some protection during radiation. This explanation is unlikely, since the whole toxin which contained a greater percentage of heavier particles than the supernatant, was more sensitive to irradiation than the supernatant fraction. There was a less orderly decrease in toxicity of both fractions and of the whole toxin than was observed in either of the first two experiments.

Earlier studies in our laboratory showed that large dosages of gamma irradiation were necessary to inactivate unpurified *Cl. botulinum* toxin in broth. The smaller irradiation dosages needed for the inactivation of the purified toxin may be a measure of the protection offered by the other constituents in crude toxin. Carpenter *(1)* showed that dialyzed toxic bacterial filtrates were more sensitive to irradiation than undialyzed filtrates.

Similar inactivation patterns were reported by Morgan *(2)* and Reed *(4)* on irradiation studies with spores of *Cl. botulinum*. However, purified toxin of type A *Cl. botulinum* in 0.05M phosphate buffer solution at pH 7.5 was not as resistant to irradiation as the spores or as unpurified toxin in broth. This parallels the difference between the thermal resistance of *Cl. botulinum* toxin and spores.

SUMMARY

In each of three experiments approximately 15 mg. of purified type A *Clostridium botulinum* toxin in 5 ml. of 0.05M sodium acetate-acetic acid buffer was placed in a Visking casing and dialyzed against 0.05M sodium phosphate buffer solution at pH 7.5. The dissociated toxin was spun in an ultracentrifuge at 100,000 times gravity for 3 hours at 0° C. to separate it into two components.

Appropriate dilutions of the whole toxin and of each of the components were irradiated in sealed glass vials at several dosage levels with Cobalt-60 to establish their inactivation patterns. The radiation levels required to inactivate the whole purified toxin and the two components separated by ultracentrifugation were established by intraperitoneal injection of appropriate dilutions of the toxin into 16 to 20 gram white mice.

There was little difference in the inactivation patterns of whole purified toxin and of the two fractions. In comparable samples with an initial toxicity of 100,000 MLD per ml., about 80% of the toxicity was removed by only 3,000 rads, whereas it required 900,000 rads to inactivate 99.99% of the toxin.

The purified toxin was approximately 100 times less resistant to radiation with Cobalt-60 than unpurified type A *Cl. botulinum* toxin in sterile trypticase broth or heat-treated surface ripened cheese. The smaller radiation dosage needed for inactivation of purified toxin may be a measure of the protection offered by the other constituents in crude toxin.

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ORGANOLEPTIC EVALUATION OF THE COMBINED EFFECTS OF HEAT AND RADIATION ON CANNED PEAS[a, b]

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At present, an irradiation dose of approximately 4.5 megarad is recommended for the safe sterilization of non-acid foods *(8)*. In this dose range the palatability of many foods may be adversely affected. Recently, several investigators *(2, 3, 4, 6)* have shown heat and gamma radiation to be synergistic when used for killing spores of anaerobic bacteria. Perhaps a combined irradiation-heat process would result in an improved product. The present report compares the organoleptic acceptability of canned peas that have been processed by (a) heat, (b) irradiation, and (c) irradiation-heat. Effects of type of closure, variety of peas, amount of brine, freezing, irradiation dose and chemical additives were investigated concurrently.

METHODS AND MATERIALS

General procedure. A blend of peas of sieve sizes 2, 3 and 4 in 307 x 306 "C" enamel cans were used. The conventional processing procedure for peas, which includes a hot water blanch, was employed through the filling stage. Most variations employed in this study were made after the filling stage and will be indicated as each experiment is presented. After closing, the samples were appropriately processed. The samples to be irradiated were packed with ice and transported by car to the Materials Testing Reactor where they were radiated within 5-15 hours after packing. Samples to be combination processed were returned in ice and heat treated within 20-24 hours after packing. The peas were evaluated initially and after 3 and 6 months of storage at 85°F.

Sterilizing processes. (a) *Heat.* The thermal process for fully-brined samples of peas was 16 minutes at 252°F., the vacuum-packed peas with little or no brine were heat processed for 20 minutes at 252°F.

(b) *Irradiation.* An irradiation dose of 4.65 megarad[c] of gamma radiation was employed.

(c) *Irradiation-Heat.* According to Kempe, Graikoski and Bonventre *(4)* the preheat radiation dose must be at least 0.93 megarad to sensitize spores of *Clostridium botulinum* to subsequent heat and lower the F_0 required. Therefore, for the combination treatment a radiation dose of 1.395 megarad followed by a thermal process of 11 minutes at 252°F. for fully-brined samples or 14 minutes at 252°F. for low-brined samples was employed. The given thermal processes result in the samples receiving 50% of the sterilizing value (F_0) provided by the thermal process used where that alone was the sterilizing agent.

Organoleptic evaluation and statistical analysis. A panel of 9-10 judges rated each sample as to flavor, texture, color and odor according to the 9-point hedonic scale *(7)*. A score of 9 was considered excellent while 5 was just acceptable. At any given storage period, the comparison of samples was conducted 4 times, giving a total of 32 to 40 judgements for each variable. Mean scores in the tables represent the average of

[a] Presented before the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, Quartermaster Research and Engineering Command, U.S. Army, and has been assigned number 888 in the series of papers approved for publication.

[c] A rad is defined as the absorption in one gram of the material being irradiated of 100 ergs of radiant energy.

all the individual values for the 3 storage periods or a total of 96 to 120 judgements made on 12 samples of each variable.

The data were statistically treated by an analysis of variance. For this presentation the average mean scores for each storage period were employed to determine the differences between samples and between storage periods. The least significant differences required at the 5% and 1% levels of probability were determined and are presented in the appropriate tables.

EXPERIMENTAL AND RESULTS

Effects of type of closure and method of sterilization. The peas were packed in a conventional manner and divided into 2 lots. One lot was closed at a product temperature of 120°F., the other lot was closed in a vacuum. Equal portions of each lot were sterilized by the 3 methods. A duplicate series of the conventionally closed and irradiated samples were made to check the ability of the panel to evaluate similar samples.

Results of the organoleptic examination are presented in Table 1. With the exception of texture, the type of closure used had no effect on the organoleptic acceptability of the peas. The texture of thermally-processed peas was superior when conventionally packed; the irradiated peas were better when vacuum packed.

TABLE 1

Organoleptic evaluation of heat processed, irradiation processed and irradiation-heat processed peas following conventional and vacuum closure.

Factor judged	Thermal 16 min. at 252° F.		Irradiation 4.65 megarad			Combination 1.395 megarad + 11 min. at 252° F.		Least significant differences	
	Conventional	Vacuum	Conventional	Conventional	Vacuum	Conventional	Vacuum	P = .05	P = .01
	Mean scores [1]								
Flavor	6.85	6.65	3.20	3.31	3.22	4.39	4.18	*0.46*	*0.64*
Texture	6.53	6.12	4.81	4.80	5.13	5.36	5.21	*0.16*	*0.22*
Color	6.54	6.63	3.23	3.34	3.55	4.04	3.79	*0.93*	*1.31*
Odor	6.42	6.69	3.26	3.28	3.12	4.33	4.15	*0.40*	*0.56*

[1] Values are averages for all individual scores for the three storage periods.

It is evident, however, that the method of sterilization was important in determining the acceptability of the final product. In all respects the thermally-processed peas were better than peas processed by the other methods. Furthermore, peas sterilized by the combined irradiation-heat process were superior to irradiated peas in regard to flavor, odor and, in general, texture, but were similar in color.

Flavor, color and odor of the peas did not change during storage at 85°F.; however, the texture decreased in acceptability during the first three months.

Effects of various amounts of brine and the method of sterilization. The cans were filled with peas and divided into 2 lots. One lot was filled with brine (160 ml. per can); in the other lot 40 ml. of brine was added to each can. The amount of salt and sugar per can was kept constant. After vacuum closure, equal portions of each lot were processed by the three sterilizing methods.

Results (Table 2) indicate that the amount of brine was important only when radiation was the method of sterilization. In this case the flavor, texture, and odor of irradiated peas were more acceptable when a full brine was used. Regardless of the process, mean scores for the fully-brined samples were generally higher.

Thermally-processed peas were superior in all respects. Peas sterilized by the combined process were superior to irradiated peas in flavor, odor and, in general, texture, but were similar in color.

Flavor, color and odor of the peas did not change during storage at 85° F.; however, the texture decreased in acceptability during the first 3 months.

Effects of variety of peas and method of sterilization. A canning variety of peas (Perfection) was compared with a freezing variety (Wyola). Samples were all packed with 40 ml. of brine and vacuum closed. Equal portions of each variety of peas were processed by the 3 sterilizing methods.

TABLE 2

Organoleptic evaluation of heat processed, irradiation processed and irradiation-heat processed peas vacuum packed with various amounts of brine.

Factor judged	Thermal at 252° F. 20 min. 16 min.		Irradiation 4.65 megarad		Combination 1.395 megarad plus 14 min. 11 min. at 252° F. at 252° F.		Least significant differences	
	(Brine)	40 ml.	160 ml.	40	160	40	160	P = .05	P = .01
				Mean scores [1]					
Flavor		6.72	6.72	2.72	3.27	4.46	4.26	*0.26*	*0.36*
Texture		5.88	6.28	4.56	5.10	5.07	5.32	*0.32*	*0.45*
Color		6.17	6.68	3.37	3.96	3.41	4.33	*1.00*	*1.42*
Odor		6.38	6.61	2.69	3.34	3.97	4.30	*0.48*	*0.68*

[1] Values are averages for all individual scores for the three storage periods.

TABLE 3

Organoleptic evaluation of canning and freezing varieties of peas that have been heat processed, irradiation processed and irradiation-heat processed.

Factor judged	Thermal 20 min. at 252° F.		Irradiation 4.65 megarad		Irradiation + heat 1.395 megarad plus 14 min. at 252° F.		Least significant differences	
	Canning	Freezing	Canning	Freezing	Canning	Freezing	P = .05	P = .01
			Mean scores [1]					
Flavor	6.88	4.59	3.23	3.20	4.73	4.06	*0.60*	*0.86*
Texture	6.32	4.35	4.74	4.43	5.18	4.43	*0.18*	*0.26*
Color	6.51	4.54	3.32	4.17	3.84	3.36	*0.75*	*1.07*
Odor	6.66	4.35	3.12	3.52	4.24	3.94	*0.55*	*0.78*

[1] Values are averages for all individual scores for the three storage periods.

Results (Table 3) indicate that the variety of peas influenced the acceptability. When both varieties of peas were heat processed, the canning variety was superior in all respects. This was expected since freezing varieties are generally not highly regarded for canning. The flavor and texture of the canning variety of peas were superior when combination processed. When the peas were sterilized by irradiation the freezing variety had a better color but the canning variety had a better texture.

Since there was an interaction between variety of peas and treatment, it will be best to consider the effects of the method of sterilization on each variety separately. First, considering only the canning variety, the thermally-processed peas were superior in all respects. Also, the combination-processed peas were better than irradiated peas in flavor, texture, and odor, but were similar in color.

In the case of the freezing variety of peas a different reaction was noted. All samples were similar in texture. The flavor and odor of thermally-processed peas were superior to the irradiated peas; the combination-processed samples were rated in between. The color of the thermally-processed and irradiated samples were similar and both were better than the combination-processd peas.

The texture of the peas progressively decreased in acceptability during storage at 85° F. No other storage effects were noted.

Effects of radiation dose and the amount of brine. The peas were canned with various amounts of brine, vacuum closed and irradiated at 0.93, 1.395, 2.79 and 4.65 megarad, respectively. Irradiation doses of 0.93 and 1.395 megarad were not sufficient to sterilize the product since many of the samples spoiled. Therefore, they were not evaluated organoleptically. An analysis of the headspace gas from some of these spoiled cans showed 75-91% carbon dioxide, 7-16% hydrogen and no oxygen. A bacteriological examination showed the presence of spore-forming rods capable of producing gas anaerobically at 30° C. The organisms were not further identified. A putrefactive odor and a greener color was noted in these samples.

Omitting, for the present, peas packed with a full brine it can be seen from Table 4 that there was no significant differences in mean scores for samples packed with 40 ml. or

TABLE 4

Organoleptic evaluation of peas vacuum packed with various amounts of brine and irradiated at 2.79 and 4.65 megarad.

Factor judged	2.79 Megarad			4.65 Megarad		Least significant differences	
	(Brine)	None	40 ml.	160 ml.	None	40 ml.	P = .05	P = .01
		Mean scores [1]			*Mean scores* [1]			
Flavor		4.77	4.84	5.40	3.97	3.97	*0.42*	*0.61*
Texture		5.08	5.30	5.79	4.40	4.73	*0.42*	*0.61*
Color		5.38	5.15	6.04	3.28	3.13	*0.47*	*0.69*
Odor		4.47	4.48	5.32	3.58	3.48	*0.37*	*0.53*

[1] Values are averages for all individual scores for the three storage periods.

no brine at each dosage level. Also, the peas irradiated at 2.79 megarad were superior in every respect to peas irradiated at 4.65 megarad.

Of the samples irradiated at 2.79 megarad the fully-brined peas were superior to peas packed with little or no brine. Similar results for samples radiated at 4.65 megarad were shown earlier (Table 2).

During storage at 85° F. the flavor, texture and color acceptability decreased for the first 3 months and then stabilized. The odor was not affected by storage.

Effects of physical state during radiation and the method of sterilization. Effects of radiating in the frozen and non-frozen condition on the acceptability of peas was determined. Samples were prepared with 40 ml. of brine, vacuum closed and divided into 2 lots. One lot was frozen and the other lot was refrigerated before and during radiation. An equal number of samples from each lot were then appropriately processed.

The mean scores (Table 5) show that there was no advantage in radiating peas in the frozen as opposed to the non-frozen state.

In all respects thermally-processed were superior to combination-processed peas and both were superior to irradiated peas. Storage at 85° F. for 6 months had no effect on the acceptability of the peas.

Effects of selected chemical additives in preventing damage during radiation. Sulfur compounds are radiosensitive, and the addition of free radical acceptors such as ascorbic acid have been used without success in reducing the off-flavors and off-odors caused by these compounds. A new approach of masking these sulfhydryl groups by specific chemical reactions rather than protecting them by competing reactions has recently been reported *(1, 5)*. A number of compounds, including pyruvic acid and pyruvaldehyde, were more effective than the addition of free radical acceptors in preventing hydrogen sulfide formation in solutions of glutathione and cysteine during irradiation.

Consequently, an experiment was carried out to test the effectiveness of a free radical acceptor (ascorbic acid), pyruvic acid and pyruvaldehyde in preventing the formation of off-flavors and off-odors in irradiated peas. Samples were packed in which the brine contained the chemical additives. The brine in the control lot contained no additive. The brine of other lots contained either 0.2% by weight of ascorbic acid, 0.2% by volume of

TABLE 5

Organoleptic evaluation of heat processed, irradiation processed and irradiation-heat processed peas as affected by physical state during irradiation

Factor judged	Thermal 20 min. at 252° F.		Irradiated 4.65 megarad		Combination 1.395 megarad + 14 min. at 252° F.		Least significant differences	
	Frozen	Non-frozen	Frozen	Non-frozen	Frozen	Non-frozen	P = .05	P = .01
			Mean scores [1]					
Flavor	6.57	6.41	3.21	3.61	4.97	4.75	*0.68*	*0.97*
Texture	6.06	5.91	4.86	4.84	5.62	5.49	*0.32*	*0.45*
Color	6.50	6.60	3.49	3.48	5.11	4.49	*0.81*	*1.16*
Odor	6.25	6.30	3.01	3.24	4.88	4.78	*0.45*	*0.63*

[1] Values are averages for all individual scores for the three storage periods.

pyruvic acid or 0.05% by volume of pyruvaldehyde. The amounts of pyruvic acid and pyruvaldehyde used were the minimum amounts which a taste panel could readily detect in the brine. All cans were vacuum closed and radiated at 4.65 megarad.

Results on the use of additives are presented in Table 6. As expected, no differences were found in the texture and color of the peas. However, peas packed in a brine containing ascorbic acid had a better flavor than those peas to which pyruvaldehyde was added. Furthermore, the odor of the irradiated control and ascorbic acid treated samples was significantly better than the odor of peas packed in a brine containing pyruvic acid or pyruvaldehyde. All organoleptic factors were affected by storage.

TABLE 6

Organoleptic evaluation of irradiated (4.65 megarad) peas packed in brines containing chemical additives

Factor judged	Additive used				Least significant differences	
	None	Ascorbic acid	Pyruvic acid	Pyruvaldehyde	P = .05	P = .01
	Mean scores [1]					
Flavor	4.37	4.52	4.33	4.02	*0.36*	*0.55*
Texture	4.83	4.97	5.02	5.04	—	—
Color	4.31	4.46	4.53	4.35	—	—
Odor	4.34	4.36	4.06	4.04	*0.19*	*0.29*

[1] Values are averages for all individual scores for the three storage periods.

DISCUSSION

Mean scores indicated that irradiated and combination-processed peas were unacceptable to the panel. However, the results did show that combination-processed peas were significantly better than irradiated peas in most cases. Radiation dose and the amount of heat used in this study for the combination treatment are probably both higher than is necessary. Kempe, Graikoski and Bonventre *(4)* using meat inoculated with spores of *Cl. botulinum,* have shown that following a radiation dose of 0.93 megarad the amount of heat required to sterilize the meat is somewhat less than 50% of that needed when radiation is not used. Therefore, by using less heat and less radiation a more acceptable product may be obtained.

Two undesirable reactions took place during storage at 85° F. First, there was a slight to moderate removal of zinc from the "C" enamel in cans sterilized by irradiation. Enamels of cans that were heat or combination processed were not affected. Second, a white precipitate that adhered to the lower end of the cans was observed in radiated and combination-processed samples, but not in the thermal controls. The amount of this precipitate was greater in irradiated samples.

Radiation of fully-brined samples resulted in the formation of hydrogen which caused the cans to swell during radiation. Consequently, it will be necessary to close the cans with sufficient vacuum to counteract the amount of gas produced, since a swollen can is unmerchantable.

SUMMARY

Acceptability of canned peas processed by (a) heat, (b) irradiation. and (c) irradiation-heat was compared. Thermally-processed peas were superior in all respects. Combination-processed peas were superior to irradiated peas

in flavor, texture, and odor, but were similar in color. In every case, except for texture, mean scores for the radiated and combination-processed peas were judged unacceptable. Flavor, color and odor remained constant during six months of storage at 85° F., but the texture acceptability decreased during the first three months.

Several other variables were investigated concurrently. The type of closure affected the texture of the peas. The amount of brine employed was important only when radiation was the method of sterilization. In this case irradiated peas were more acceptable when a full brine was used. In general the canning variety of peas was superior to the freezing variety. However, the freezing variety was less affected by the method of sterilization.

Peas irradiated at 2.79 megarad were more acceptable than peas radiated at 4.65 megarad in all respects. There was no advantage in radiating peas in the frozen state as opposed to the non-frozen state. Presence of ascorbic acid, pyruvic acid or pyruvaldehyde in the brine was ineffective in preventing the formation of off-flavors and off-odors during radiation.

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THE FLAVOR OF CANNED PEAS IN RELATION TO THEIR PYRROLIDONECARBOXYLIC ACID CONTENT AND TO CERTAIN OTHER FACTORS[a, b]

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The process of canning brings about noticeable changes in the flavor of peas. The vegetable prepared in this fashion no longer has the flavor and color of the home-cooked fresh vegetable. The present study was undertaken to investigate certain chemical changes taking place throughout the period of the high temperature cook during processing, which might account for these flavor differences.

It is known that pyrrolidonecarboxylic acid (PCA) can be formed at sterilization temperatures from glutamine *(1, 10, 11)*. Previous work at this institution showed that it is present and can contribute to an off-flavor in canned beet products *(10)*. It was also shown to be present in freshly processed tomato juice which, after lengthy storage, shows a decided increase in the content of this compound *(8)*. Further, it is known that under certain conditions with pressure, fats can hydrolyze, liberating the free fatty acids *(2)*. The pressures of the canning process, however, are considerably lower than normally required to bring about this reaction. These possible contributors to the change in flavor in peas during the canning process were explored in the present work.

MATERIALS AND METHODS

Five samples of canned peas of varying quality were obtained on the market. In addition, a lot of frozen blanched Perfected Freezer variety of peas was canned in the usual fashion. Another sample of this same lot was prepared for analysis without canning. This latter gave a control on the material before processing by high temperature sterilization.

Pyrrolidonecarboxylic acid was determined by extraction with ethyl acetate at pH 2.5, hydrolyzed to glutamic acid, and measured as such *(9)*.

Presence of PCA was further determined as follows. Formic acid was determined at the same time. A slurry of the contents of each can of peas was prepared by whipping in a Waring blender. It was frozen and dried by lyophilization. Samples of the lyophilized material equivalent to 1 g. of each slurry were weighed and treated with 5 ml. of distilled water. The mixture was heated on a steam bath for 15 minutes, followed by centrifugation at 2000 r.p.m. for 10 minutes. Supernatant was saved, and the residue was treated with 5 ml. of distilled water again, and treated as before. This was repeated two more times. Collected supernatants were concentrated to a volume of 0.5 ml. on the steam bath, then treated with 1 gram of silicic acid followed by careful mixing. Finally, 0.5 ml. of 0.5 N H_2SO_4 was added, and the whole carefully mixed. This was dry enough to pour into a silicic acid column prepared with 8 g. of silicic acid, 5 ml. of 0.5 N H_2SO_4, and 60 ml. of $CHCl_3$. The technique of Bulen, Varner, and Burrell *(3)* was employed.

A sample of the lyophilized slurry was extracted with anhydrous ethyl ether in a Soxhlet extractor. The ether was evaporated under reduced pressure to obtain the lipid

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[b] Presented at the Eighteenth Annual meeting of the Institute of Food Technologists, May 27, 1958.

material. The total acid of this extracted material was determined as described in previous publications *(5, 6)*.

Taste tests were run by a panel of 6 members previously familiarized with the PCA flavor.

RESULTS AND DISCUSSION

Table 1 shows the results of the extraction of PCA with ethyl acetate from various samples of canned peas, followed by determination as glutamic acid. It is obvious that PCA is present in the canned peas. It is absent, as noted in the method described below, from the peas which were blanched only. This suggests that this substance, because of its peculiar flavor, could influence the flavor of the canned product.

TABLE 1

Analysis of canned peas for variations in pyrrolidonecarboxylic acid content

Pea sample	pH	Mg. PCA [1] in 100 g. slurry
A	6.12	19
B	5.95	115
C	6.00	25
D	6.12	97
E	6.01	32
F [2]	6.25	16

[1] Mean of triplicate determinations on duplicate samples. Hydrolyzed and measured as glutamic acid.
[2] Perfected Freezer blanched peas, canned in the usual fashion, previous to analysis.

The method involving the use of the silicic acid column confirmed these findings, as far as the PCA is concerned. It showed, also, the absence of this substance in the peas which were blanched before storing at 0° F. Furthermore, this method shows tentatively that formic acid is present in the canned peas also, but in small quantity. Organoleptic tests indicated that formic acid did not contribute any noticeable flavor to the product, and, therefore, while it may be indirectly and remotely involved in the flavor of canned peas, certainly it cannot be considered of any importance, at least as indicated by the results of the present work.

Determination of the titratable acid of the lipid extracted from the lyophilized material showed an acid number of 6.4. This value is substantially the same as that found by Lee *et al. (7)* for fresh and blanched peas, and indicates that the canning process did not liberate such compounds from the lipid material in the peas.

Results with the taste panel showed that no apparent correlation between canned pea flavor and PCA concentration existed. However, in view of the fact that a sample of canned peas which did not contain PCA was not available, it was impossible to make an absolute evaluation of the specific role of this compound in this product. Blanched peas used as controls possess a fresh flavor which affects the judges' ability to taste PCA in peas in relation to canned flavor. It is apparent, therefore, that the evidence does not warrant the conclusion that PCA content is necessarily a predominant factor in the distinctive flavor of canned peas. Samples with the highest PCA concentration could be distinguished from the others, however. Five out of 6 members placed the highest PCA sample at the poorest end of the rating scale. It is

well to note that the one member of the panel that gave this sample the highest rating prefers the flavor of canned peas in the choice between canned peas versus fresh or frozen peas.

Results of the triangle intensity test (Table 2) show that PCA (as the

TABLE 2

Triangle-intensity panel scores for PCA added (as the ammonium salt) to blanched peas

Sample [1] mg. PCA	Panel Score
200	22.2**
150	22.2**
100	50.5

[1] In 100 grams of blanched frozen peas which had been cooked for this test.
** Significant at the 1% level.

ammonium salt) could be significantly detected when the PCA concentration exceeded 100 mg. per 100 g. of slurry. From these data we conclude, therefore, that while PCA is formed during the canning of peas, and possesses considerable flavor (in contrast with its probable precursor, glutamine) it is only one component of canned pea flavor and not at all times apparently, an important one.

SUMMARY

It was shown that pyrrolidonecarboxylic acid is present in canned peas. It was further shown organoleptically that the presence of PCA could be detected significantly in cooked peas when the concentration exceeded 100 mg. per 100 g. of pea slurry.

It was concluded that while PCA is formed during the canning of peas, it is only one component of canned pea flavor and not at all times, apparently, an important one.

The presence of salts of formic acid in canned peas was noted, but concentration was too low to be of any apparent direct importance.

It was found that free fatty acids are not formed from the lipids during the canning process.

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VOLATILE REDUCING SUBSTANCES AND THE EVALUATION OF THE AROMA OF COFFEE[a]

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Since the work of Bernheimer and of Erdmann, cited by Punnett *(10)*, on the analysis of the coffee roaster gases and of steam distillates of roasted coffee, a number of reports on the composition of coffee during the processing stages have been presented. Among these are those of Clements and Deatherage, *(1)* and of Hughes and Smith *(6)*. The chemistry of coffee has been reviewed recently by Lockhart *(7)* and by Reichstein and Staudinger *(11)*.

Several investigations to evaluate chemically the intensity of coffee aroma or savor and of the effects of the processing operations thereon have been published in recent years. Högl and Wurziger *(5, 14)* estimated the total content of aromatic substances in coffee by determining the oxidizability of steam distillates by an acid potassium dichromate solution. The latter expressed his results as "volatile values" and showed a variation in values for different coffees. Natarajan *et al (8)* used a modification of a procedure described by Farber *(2)* and reported on the variation in "volatile values" of a number of Indian coffees. They used an alkaline potassium permanganate solution as oxidizing reagent at a temperature of 105°C. and a reaction time of 2 hours. Greenbaum *(4)* used the oxidizability of steam distillates of coffee and coffee extracts by acid potassium permanganate as a measure of the aroma and flavor. O'Meara *et al (9)* determined the electron paramagnetic resonance of coffee and suggested its usefulness in studying the roasting operation. Rhodes *(12)* in a study of the volatile constituents of coffee by gas chromatography found that various coffee roasts and coffee brands could be distinguished and quantitatively evaluated by variations in the individual vapor chromatograms. Van den Dool *(13)* reported that natural coffee extracts had a characteristic ultraviolet absorption at 275 mμ, which could be used to detect the presence of coffee substitutes and to measure the amount of natural coffee aroma in a preparation.

In this report a rapid and relatively simple procedure is presented as a means of obtaining a quantitative and objective evaluation of the aroma of coffees and of the changes which occur during processing and subsequent storage. The determination is carried out at room or ambient temperature and under conditions approximating those existing during the usual organoleptic judgment.

MATERIALS AND METHODS

Coffees. Various commercially available coffee brands, purchased in local markets, were freshly opened at the time of the examinations. All were vacuum packed cans except number 3, which was in a paper bag. All were classed as "drip" grinds. The

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different roasts of coffee were obtained from 3 sources; one coffee importing house and 2 commercial coffee producers. The types of roasts were based on the opinions of the coffee experts at the various establishments. The various experimental samples were brought to the laboratory as soon as possible after their preparation and were analyzed within an hour. The ground coffee brews were tested similarly to the customary organoleptic procedure. One measure of ground coffee was placed in a cup and freshly boiled tap water was added to fill the cup. The mixture was stirred with a spoon and submitted to sensory evaluation. Ten ml. of the hot supernatant extract was taken for the analysis. For the instant powdered and liquid concentrated coffees, one and two teaspoons, respectively, were added to a cup and freshly boiled tap water was added to fill the cup. After mixing and sensory evaluation, 10 ml of the hot solutions were taken for analysis.

Volatile reducing substances. VRS content of the samples was determined in the recirculating system previously described *(3)*. 0.1 N potassium permanganate in N sodium hydroxide solution was used as the oxidizing reagent.

For the various aeration time intervals of the samples, the reaction flask with the 10 ml. of oxidizing reagent was serially changed after successive increasing times, using the same sample of coffee in the aeration flask.

RESULTS

Course of the aeration. Representative aeration curves for a ground coffee and for a coffee brew are shown in Figure 1. Volatilization of the constituents in air at a temperature of about 23°C (73.4°F.) with time was progressive and tends to a maximum value. However, during the rather short aeration times used in this study the maximum values were not reached. Nearly complete removal of the volatile components in a sample was neither attempted nor obtained. The VRS values which are reported in this study are therefore relative and not definitive for any sample or type of material.

VRS in commercially available coffees. The VRS content of 8 different brands of ground coffee is given in Table I, after two aeration times. It was evident that a varia-

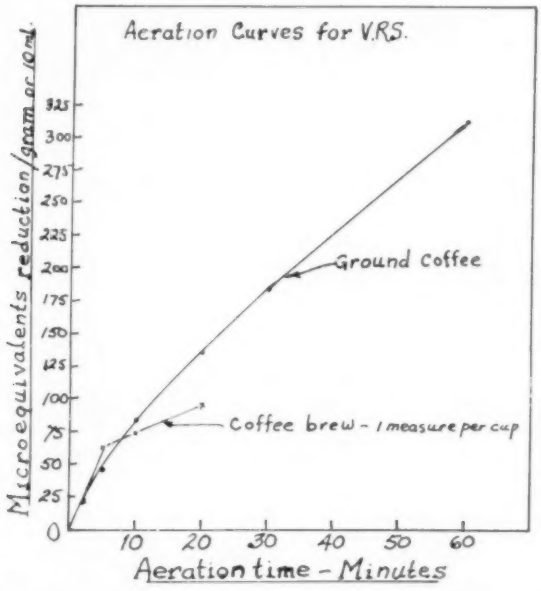

Fig. 1. Aeration curves for a ground coffee sample and for a ground coffee brew.

tion in VRS content existed between various coffees. Furthermore, differences in composition of the coffees were indicated by the relation between the VRS volatilized after 10 and 30 minutes. This observation suggested that this procedure might offer a further means of comparing the relative kinds of aromatic constituents in various samples. No sensory judgments were included for the coffees of Table 1, even though differences in aroma between the various brands were discernable. It was felt that without an expert analysis the judgments would not be very significant.

Relation between roasting and VRS content. Three different series of experiments were carried out in order to determine whether there was any relationship between the stage of roasting of the green coffee beans and the resulting VRS content. All 3 roasting runs were made in different establishments by separate coffee experts. As a result, the various roasting stages were not strictly comparable. The normal or optimum stages varied from a medium brown color to that of a dark brown color. What one expert called a normal roast was closer in color to that called a dark roast by another. Therefore, the data from the 3 series of roasted coffees were only relative among themselves, and not strictly comparable with those from other series. VRS data for various roasts of 5 green coffees from different sources are given in Table 2. The change in VRS content with the stage of roasting was evident. In this case all the "dark" roasts had a smaller VRS content than the "normal" roasts, but a greater VRS content than the "light" roasts. In Table 3 is shown the content of VRS of a green coffee sample during roasting, for progressive intervals of aeration. In this series there was a progressive increase in the VRS values for the coffee at the different roasting stages. Results of the third roasting series are given in Table 4. Similar to the results of Table 1, VRS value reached a maximum at what was judged the optimum roast and then decreased on further roasting. Organoleptic differences between the various roasts were also quite distinct.

Changes in VRS during storage. A commercial coffee sample was stored at various temperatures in a household-type refrigerator, at an intermediate temperature and at ambient laboratory temperature. VRS contents after increasing storage times at the

TABLE 1

VRS content of various coffee brands [1]

Sample number	VRS per gram ground coffee after minutes aeration	
	10	30
1	66.7	183.5
2	39.7	81.0
3	37.5	—
4	70.6	158.0
5	98.0	170.0
6	108.0	214.0
7	47.7	105.0
8	65.0	140.0

[1] All brands were "drip grind" and freshly opened for sampling.

TABLE 2

VRS in various types of roasts [1] of different coffees

Sample of coffee	VRS per 2 grams after 20 minutes		
	Light roast	Normal roast	Dark roast
Brazilian Santos	83.8	211.9	158.8
Indonesian Robusta	100.2	164.2	165.2
Mexican	135.5	166.0	111.2
African Tanganyika	192.0	204.0	193.2
Hawaiian Kona	197.0	262.0	217.0

[1] The grading of the roasts was made by an experienced coffee taster of an importing house. The colors of the roasts were shades of light tan brown, brown and a dark brown respectively. The roasting was in a laboratory-scale gas-fired roaster.

TABLE 3

VRS in various types of roasts [1] of a Guatemalan coffee

Sample description	Sensory findings	VRS per 2 grams after minutes			
		5	10	20	30
"Very light"	light yellow brown color—no definite coffee aroma	8.6	15.3	29.6	50.6
"Normal"	brown color—definite, coffee aroma	44.0	76.5	140.5	207.0
"Dark"	darker brown color—coffee aroma and sharper odor	64.0	103.0	181.0	266.0

[1] The roasting and grading was done by an experienced coffee taster from a commercial coffee producer.

TABLE 4

Effect of roasting time on the VRS content of an Amenia Colombian coffee [1]

Roasting time	Sensory findings	Shrinkage	VRS after minutes aeration		
			10	20	30
Min.		%			
5	mild pleasant aroma — no sharpness	10.4	76.4	160.5	218.5
6	pleasant aroma with some sharpness	12.5	131.3	207.5	259.0
6.16	"optimum roast" pleasant aroma with definite marked sharpness	13.0	148.0	278.0	380.0
7.25	not pleasant — solvent-like very sharp	14.8	113.7	216.0	308.5

[1] Two grams coffee were aerated into 0.1 N $KMnO_4$ in N NaOH. The roasting and grading for optimum roasting time was made at a commercial coffee producer.

TABLE 5

VSR content of a commercial coffee during storage at various temperatures

Storage temp. °F.	VRS per gram after days storage			
	0	1	5	30
40 (4.4° C.)	—	214.0	173.0	151.5
65 (18.3° C.)	214.0	—	139.0	105.0
75 (23.9° C.)	214.0	—	115.9	80.0

30 Minutes aeration time. At 75° F. a rancidity was noticeable after 30 days.

different temperatures were determined periodically. Results are presented in Table 5. The content of VRS decreased by about 30% after 30 days at 40°F. ((4.4°C.), by 51% at 65°F. (18.3°C.) and by 62% at 75°F. (23.9°C.). The coffee after 30 days at 40°F. still had a definite, though weak, coffee aroma. The coffee after 30 days at 65°F. had a somewhat flat odor resembling coffee, but not very pleasant, with a noticeable more predominant burnt odor. The coffee after 30 days at 75°F. had a definite somewhat rancid odor along with a burnt odor and practically no pleasant coffee aroma. The beneficial effect of the low temperature storage on the retention of the aroma was quite evident from this series of tests.

VRS content of coffee brews. In Table 6 are shown the results of a series of tests on the VRS contents of brews from a number of coffee samples. The deleterious effect of storage, particularly at the higher temperature, on the content of VRS and on the sensory judgment was evident. The difference in VRS content between the brew from the fresh ground coffee and that from the instant coffees was also of interest. A parallel between the presence of a characteristic coffee aroma and flavor and the content of VRS was also indicated by the data.

TABLE 6

VRS content of brews [1] of various coffees

Sample description	VRS per 10 ml. after minutes aeration			Sensory findings
	5	10	20	
Freshly opened — drip grind	65.2	78.2	97.8	Good aroma and flavor
After 8 months at 40° F (4.4° C)	17.4	30.4	36.9	Bland odor — watery, sl. burnt flavor
After 8 months at 75° F (23.9° C)	10.9	19.5	26.0	Tallowy rancid odor — a bitter tobacco-like flavor
Instant powder	37.8	41.3	45.5	Mainly a bitter roasted flavor & burnt odor
Liquid Coffee Concentrate	21.7	34.7	45.5	Somewhat burnt odor — bland & sl. bitter flavor

[1] One measure of ground coffee was dispersed in a cup of hot water and the extract was sampled: 1 tsp. of powdered instant and 2 tsp. of liquid concentrate of coffee were diluted to a cup with hot water.

DISCUSSION

The main purpose of the present report was to bring to the attention of coffee investigators and other interested persons the potential usefulness of the VRS procedure as a quantitative means to evaluate coffee aroma, supplementary to, and also complementary to, the widely used and standard organoleptic judgment. The data of the tables definitely showed that this aim was possible. The correlations between sensory judgments and VRS contents were definite. They offered the possibility of establishing working standards or levels by which to compare different samples and treatments. They also definitely pointed out the subjective nature of the presently accepted organoleptic grading system. The varying interpretations of the coffee roasting process, for example, by individual coffee experts, was also clearly evident from the above results. A quantitative means of checking the sensory evaluations could prove of definite value in the establishment of desired reference points in any one institution and perhaps in the coffee industry in general.

The apparent benefit for the retention of coffee aroma of a low temperature storage of ground coffee (even in a closed container), as indicated by the data of Table 5, was of interest. In this connection, the noticeable escape of pleasing coffee aroma on opening a room temperature-stored vacuum-packed can of coffee is a well-known fact. When an unopened can was stored in a household refrigerator overnight or longer before being opened, the escape of coffee aroma was markedly reduced.

It is possible that with further studies, among others, on the effect of low temperature storage of ground coffee and on the use of the VRS procedure

as a quantitative guide, improved practices may be developed by the coffee roasting industry. One practical result to be sought would be the development of a technique whereby the very pleasing aroma of fresh ground coffee could be transferred to the brew made from the coffee. The availability of a ready means to quantitatively evaluate the effect of new processes on the intensity of the coffee aroma could also prove a stimulus for investigation in this direction.

A comparison of the VRS procedure with those previously mentioned as possible means of evaluating coffee aroma was of interest from a number of points of view. First, the rather cumbersome and time-consuming steam distillation was avoided. Steam distillation had a further disadvantage that substances not significantly contributing to the aroma, but yet steam volatile, were determined in the distillate. Second, acid potassium dichromate and acid potassium permanganate are known to be weaker or less efficient oxidizing agents than an alkaline potassium permanganate solution. Third, an electron paramagnetic resonance apparatus, a gas liquid phase chromatograph, and even an ultraviolet spectrophotometer are not as readily available pieces of laboratory equipment and are certainly more expensive than the glassware and the circulating pump which are required for the determination of VRS. Fourth, as was shown above, the content of VRS could be determined within a short time, if speed were a prerequisite. Finally, the relative simplicity of the VRS apparatus and of the technical details would enable determinations to be made on a routine basis by individuals with limited technical training. A knowledge of elementary chemical principles and techniques and, above all, careful, accurate and clean work habits would enable significant and reproducible results to be obtained.

SUMMARY

The content of volatile reducing substances (VRS) has been determined in eight commercial coffees, in coffees after increasing roasting periods, and after storage at various temperatures, and in brews of various coffee samples. The VRS content correlated with the organoleptic judgment of the various coffee roasts and of the coffee during storage at different temperatures. On the basis of the present data the determination of the VRS content offered a rapid and relatively simple means to evaluate the intensity of the aroma of coffee and its products.

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SOME OBSERVATIONS ON THE MODIFICATION OF FREEZE DEHYDRATED MEAT[a, b]

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Studies on the biochemistry of meat during post mortem aging has shown that the muscle plasma plays an important role in the changes in the chemical and physical properties of the meat *(6, 15, 16, 17, 25, 26, 27, 28)*. Arnold *et al. (3)* reported that during post mortem aging, sodium and calcium were continuously released by the muscle protein while potassium was absorbed after the first 24 hours. They also stated that the cationic shifts during post mortem aging appears to increase the electrical charge of the protein molecules of meat resulting in a measurable increase of hydration accompanied by improved tenderness. Wierbicki *et al. (26, 29)* established that the water holding capacity of meat may be altered by modifying the ionic atmosphere in meat. Again these authors *(29, 30)* stated that ion protein interactions are taking place during post mortem aging of meat and also during cooking process.

Preparations, properties, and histological, histochemical and some chemical characteristics of freeze-dehydrated meat have been described *(7, 19, 20, 21, 22)*. Harper and Tappel *(14)* stated that the texture of the freeze-dried meat is drier than the frozen control and that this dry texture is one of the principal problems which remains to be solved in the field of lyophilization of meat.

The studies to be reported here were directed to an improvement in the texture and toughness of cooked reconstituted freeze-dehydrated meat. Since the rehydrated meat tends to be tough and woody upon cooking, it was thought that modification of meat through vascular infusion of various salts to improve the water holding properties of the muscle protein prior to freeze-dehydration might be carried over to the cooked rehydrated meat and thereby improve the acceptability of the product. In order to evaluate the effect of modification of the meat it was also necessary to study in some detail the relationship of post mortem aging and of cooking prior to freeze-dehydration to the quality of the meat as eaten.

MATERIALS AND METHODS

Twenty-eight cattle were used in this work; most of them were comparable to lower half of U. S. good grade. The sampling techniques and methods used have been previously reported from this laboratory *(3, 6, 17, 28, 29)*.

Modification of meat: Three different methods were used for modification of meat under investigation:

1) *Modification of ground meat:* Aliquot samples of ground meat were weighed and modifier solutions were added to the ground meat using one part of additive to 10

[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 28, 1958.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 915 in the series of papers approved for publication.

parts of meat. Meat receiving equivalent amounts of distilled water were used as controls. Samples were thoroughly mixed and examined after standing at room temperature for 2 hours.

2) *Modification of whole rounds using infusion techniques:* The cattle were slaughtered and dressed in the usual manner. Carcasses were split and the hot rounds were excised and used immediately for infusion experiments. One round from each animal was weighed and then the desired amount of the modifier solution was infused using one part of modifier solution to 12.5 parts of meat; the other round remained as control. The infusion was carried out as described by Weiser *et al. (24)*. A 10-gauge needle was used to cannulate the external iliac artery and the modifier solution was infused with pressure to overcome leaking arteries which could not be closed by hemostats. Live steam was used to sterilize all equipment for the infusion experiments. Samples from both the control and infused rounds were obtained from time to time for analysis and for freeze-dehydration.

3) *Modification during the rehydration process:* This was accomplished by adding various solutes to the water used for rehydration. The freeze-dried meat was trimmed from excess fat and connective tissue, cut into small pieces, and immediately rehydrated by adding the desired amount of rehydrating solution to restore the moisture content to that originally present in the meat.

Freeze-dehydration. The meat samples were freeze-dried in a Stokes freeze-dryer with water heated shelves. Two methods were used for the freeze-dehydration:

1) The Quartermaster Food and Container Institute for the Armed Forces in Chicago freeze-dehydrated meat using the following procedure: Samples were held at -23° C. for 2-3 weeks, sawed while frozen into ½ in. thick steaks and then freeze-dehydrated over a period of 24 hours using a plate temperature of 43° C. and chamber pressure of 1500 μ of mercury. After completion of the dehydration the freeze-dried steaks were held in polyethylene bags (containing calcium oxide) at room temperature for about 2 hours and then canned under vacuum.

2) Meat was cut into ½ or ¾ in. steaks, held at -40° C. for 1 or 2 days and freeze-dried using a plate temperature of 22-30° C. and chamber pressure of 300-400 μ Hg.

Rehydration of meat. Fresh control or modified freeze-dried meat was rehydrated at room temperature for 20-30 minutes by adding the desired amount of distilled water to the dehydrated meat samples in order to obtain the same moisture content as before dehydration. Cooked control or modified freeze-dried meat was rehydrated for 30-40 min. at 40-50° C.

Cooking prior to freeze-dehydration. The meat was weighed, wrapped in parchment paper, and cooked in the oven at 121° C. to an internal temperature of 71° C. and then chilled for 12-15 hrs. The chilled meat was frozen and freeze-dehydrated after being sawed into ½ in. thick steaks.

Water holding properties. This was determined using the tube method of Wierbicki *et al. (29)*.

Electrophoresis measurements. The Perkin-Elmer model 38 A Tiselius electrophoresis apparatus with a standard 2 mm. cell was used. The electrophoresis cell was maintained at 0.5± 0.1° C. and mobilities were calculated from the velocities of the descending boundaries using the conductivities of the muscle structural protein extracts measured at 0° C. The relative areas of the components in the photograph were determined by placing the negatives on finely ruled cross section paper and counting squares. The electrophoretic properties of proteins soluble in water and veronal buffer (5.5-diethylbarbiturate pH 8.6 and ionic strength = 0.1) were studied in order to investigate the changes, if any, which may take place during the freeze-dehydration. The extracts were dialyzed, for 48 hrs., against veronal buffer pH 8.6 and ionic strength = 0.1. This ionic strength was chosen to extract most of the muscle structural proteins and to eliminate differences in the solutions in the moving boundary method and to avoid large changes in the specific conductivity during the electrophoresis experiments, especially when the protein concentration in the sample was 1% lower.

RESULTS AND DISCUSSION

Effect of post mortem aging, frozen storage and freeze-drying on the chemistry of the rehydrated meat. Carcasses of 4 steers were aged at 2-3°

C. One loin of each animal was frozen after 2 days; the other was aged for an additional period of 13 days (15 days post mortem) and then frozen. Frozen loins were freeze-dehydrated at the Qm. Food and Container Institute for the Armed Forces for 24 hrs., as described.

Since this meat was stored in the freezer for 2-3 weeks prior to freeze-drying, it was necessary to investigate the effect of frozen storage on the chemistry and quality of meat in order to understand the relative effects of post mortem aging and freeze-dehydration. Meat was examined after post mortem aging and during frozen storage; the freeze-dehydrated meat samples were reconstituted and then examined.

Although the pH of fresh meat increased slightly during aging, no changes were detected as a result of frozen storage or freeze-dehydration of the meat samples. The water soluble nitrogen content reported as percentage of soluble nitrogen extracted are shown in Table 1 and indicated that whereas

TABLE 1

Effect of post mortem aging, frozen storage and freeze-drying (43° C. plate temperature and 1500 μ Hg. chamber pressure) on water soluble nitrogen and juice expressed on heating at 70° C. Average results of 4 steers.

Sample	Before freezing	Stored frozen 25-28 days	After reconstitution
Aged 2 days			
% of total N soluble in water	23.5	23.6	14.6
Juice, % of total moisture	46.4	46.2	53.4
Aged 15 days			
% of total N soluble in water	22.8	24.7 [1]	15.5
Juice, % of total moisture	42.9	45.2 [1]	51.5

[1] Stored frozen for 14 days.

post mortem aging and frozen storage had no effect, freeze-dehydration resulted in a considerable decrease in the concentration of this protein fraction. The amount of juice released during heating the meat for 30 min. at 70° C., reported as percentage of the total moisture, was determined and the results are recorded in Table 1. Confirming previous findings of Wierbicki *et al. (29)*, the amount of juice released decreased upon post mortem aging indicating an increase in the water holding properties of the meat. Freeze-dehydration on the other hand exerted an adverse effect on the water holding properties of meat. The rehydrated meat samples released much more juice than the same samples before freeze-drying.

Shifts in calcium and magnesium were investigated in both the water extract and in the juice released (Table 2). Total calcium concentration in the water extract increased upon post mortem aging, frozen storage and freeze-dehydration. This increase in calcium was more pronounced after reconstitution of the meat samples than upon frozen storage. The ionic calcium in the water extract decreased upon frozen storage and was slightly affected by freeze-dehydration. Although the ionic calcium concentration in the juice released upon heating the meat at 70° C. for 30 min. increased upon post mortem aging, freeze-dehydration exerted no effect. The concentration of ionic magnesium in the water extract decreased as a result

TABLE 2

Effect of post mortem aging, frozen storage, and freeze-drying on the calcium and magnesium in the water extract and juice. Results reported as milliequivalents per 1 g. total N of the muscle.

Sample	Before freezing				Stored frozen 25-28 days				After reconstitution			
	Total	Ca	Ca^{++}	Mg^{++}	Total	Ca	Ca^{++}	Mg^{++}	Total	Ca	Ca^{++}	Mg^{++}
Water extract	AGED 2 DAYS											
Mean		0.11	0.07	0.64		0.18	0.04	0.57		0.24	0.08	0.42
St. dev.		0.02	0.03	0.19		0.05	0.01	0.13		0.06	0.03	0.07
Juice												
Mean		——	0.03	0.28		——	0.03	0.25		——	0.04	0.26
St. dev.		——	0.01	0.05		——	0.01	0.07		——	0.01	0.02
Water extract	AGED 15 DAYS[1]											
Mean		0.12	0.08	0.54		0.14	0.05	0.57		0.22	0.07	0.44
St. dev.		0.01	0.03	0.15		0.05	0.02	0.17		0.06	0.02	0.06
Juice												
Mean		——	0.06	0.25		——	0.03	0.25		——	0.04	0.25
St. dev.		——	0.02	0.07		——	0.01	0.05		——	0.02	0.04

[1] Stored frozen for 14 days.

of post mortem aging and freeze-dehydration at both 2 and 15 days post mortem. A slight decrease due to post mortem aging was detected in the concentration of Mg^{++} in the juice released while freeze-dehydration had very little effect.

Effect of modifying meat prior to freeze-dehydration. When it was found that the decrease in water holding properties of the rehydrated meat samples was independent of the post mortem aging, studies were directed to the modification of meat by the infusion of inorganic solutes at the time of slaughter so as to improve the texture and the water holding capacity of the meat used for freeze-drying.

Experiments using fresh ground beef indicated that the addition of sodium ascorbate as antioxidant in meat might be of value and that an additive solution containing 0.005 M Na-ascorbate and 1.03-1.38 M NaCl or 0.135-0.27 M KCl, or both, would favorably modify the physical characteristics of the ground meat. Both $MgCl_2$ and $CaCl_2$ showed remarkable effect on the water holding properties of meat but the modified product had an objectionably bitter taste especially when the concentration of these solutes exceeded 0.011 M and 0.018 M, respectively, in the additive solution. A modifier solution containing a mixture of 1.03 M NaCl, 0.0055 M $MgCl_2$, 0.009 M $CaCl_2$, 0.135 M KCl and 0.005 M Na-ascorbate was also found to be satisfactory in bringing about the desirable physical changes in ground meat (additive solution/meat = 1/10).

Infusion experiments using fresh beef rounds and various additive solutions were conducted. Results of infusing a modifier solution containing 1.03 M NaCl, 0.135 M KCl and 0.005 M Na-ascorbate (modifier solution/meat = 1/12.5) are reported. Six cattle were used for this experiment; one round from each animal was infused, the other round served as a control. Samples of the modified and control meat (aged 2 days at 3° C.) were freeze-dried

using the 2 methods of dehydration as previously described. Dehydrated samples were reconstituted and both fresh and rehydrated meat were analyzed. Results were as follows:

Freeze-dehydration using a plate temperature of 43° C. and a chamber absolute pressure of 1500 μ Hg. No effect was detected on the pH of both control or modified meat as a result of this freeze-drying. Although modification of meat through infusion had very little effect on the soluble nitrogen content in the water extract, freeze-dehydration markedly reduced the concentration of this protein fraction in both the control and the modified meat samples to about the same degree (Table 3).

Modification of the meat caused a slight positive shift in both the ionic and total calcium in the water extract apparently due to exchange with K and Na present in the modifier solution. Both total calcium and magnesium in the control and modified meat samples increased as a result of freeze-drying. The amount of juice released upon heating the meat at 40° C. and 70° C. for 30 min., reported as percent of the total moisture, for both the control and modified meat during post mortem aging and after rehydration are recorded in Table 3. Modification of meat favorably affected the water holding properties of the meat. The amount of juice released by heating the rehydrated modified meat samples at 40° C. and 70° C. was much less than the rehydrated controls indicating that the infusion of a combination of low concentrations of solutes favorably affected the water holding properties of the rehydrated meat samples. The amount of juice released upon heating the rehydrated meat (both modified and control) was much more than their respective samples before freeze-drying. This indicates that the freeze-drying process affected the water holding properties of both the control and modified meat samples resulting in a product with a dry texture. From these results it can be stated that the improvement in the water holding capacity as a result of changing the ionic atmosphere in meat did not carry over to a great extent to the rehydration of the freeze-dried meat. It seems that freeze-dehydration of meat profoundly and adversely affected the protein

TABLE 3

Effect of freeze-drying on nitrogen soluble in water and in expressed juice at 70° C. and on the amount of juice expressed at 40° C. and 70° C. Average results of three animals are reported.

	1-2 hrs. post mortem		2 days post mortem			
	Fresh		Fresh		Rehydrated	
	Control	Modified	Control	Modified	Control	Modified
Percent of total nitrogen in						
Water extract	23.4	24.3	23.9	24.0	15.7	16.7
Juice 70° C.	6.7	3.0	6.6	4.1	7.3	6.8
Percent of total moisture lost						
70° C.	44.1	14.5	43.8	33.9	59.7	51.5
40° C.	—	—	—	—	32.3	17.1

structure. The bonds that are due to electrostatic interaction between polar groups (e. g. hydrogen bond formation) and to Van de Waals forces between non-polar groups of the meat protein may be affected in some way during this freeze-dehydration process resulting in irreversible structural changes. Denaturation of the protein with resulting changes in permeability of the fibers appear to result from freeze-drying as evidenced by the decrease in the concentration of the water soluble nitrogen content, calcium and magnesium shift in the water extract and the juice, and the decrease in water holding properties of meat.

Freeze-dehydration using a plate temperature of 22-30° C. and a chamber absolute pressure of 300-400 μ Hg. No effect was detected on the pH of meat, the water, and the 4% trichloroacetic acid soluble protein of both the control or the modified meat samples (Table 4). Small changes were detected in the concentration of the calcium in the water extract and in the juice released upon heating the meat at 70° C. for 30 min. (Table 4). Slight decrease was detected in the water holding capacity of the rehydrated control or modified samples compared to the fresh meat samples. The water holding capacity of the modified rehydrated meat was much higher than that of the control meat indicating that modification through infusion of round muscles with a low concentration of inorganic solutes followed by freeze-dehydration at a lower temperature and pressure, was carried over to the rehydrated meat samples. It should be stated that the color of the rehydrated meat resembled that of the fresh meat and that the modified cooked rehydrated samples were more acceptable in tenderness, juiciness, and flavor. Since these samples were prepared in the University laboratories and over a much shorter elapsed time than when the freeze-dehydration was done in Chicago, it is not possible to say what factors contributed to the improvement. At this time it is presumed to be the lower plate temperatures.

TABLE 4

Effect of freeze-drying (22-30° C. plate temperature and 300-400 μ Hg. chamber pressure) on control and modified meat. Average results of three animals are reported.

Sample	Fresh		After rehydration	
	Control	Modified	Control	Modified
Soluble nitrogen [1]				
Water extract	27.5	25.8	26.2	25.8
Trichloracetic acid	10.7	10.5	10.6	11.7
Total calcium [2]				
Water extract	0.12	0.11	0.10	0.11
Ionic calcium [2]				
Water extract	0.08	0.06	0.05	0.06
Juice	0.03	0.03	0.02	0.02
Moisture loss [3]				
40° C.	23.3	5.5	22.0	5.6
70° C.	50.1	38.6	52.7	38.7

[1] Percent total N extracted.
[2] Milliequivalents per 1 g. total N of the muscle.
[3] Percent of total moisture.

TABLE 5

Effect of cooking prior to freeze-dehydration on some chemical and physical properties of control and modified meat. Average results of two animals are reported.

	Before cooking		After cooking			
			Chilled		Rehydration	
	Control	Modified	Control	Modified	Control	Modified
pH:						
Meat	5.5	5.6	5.7	6.0	5.8	5.9
Juice	——	——	6.0	6.1	5.8	6.1
Nitrogen [1] soluble in						
Water	20.5	21.4	5.5	6.3	7.4	8.2
Trichloracetic acid	8.5	9.0	6.0	6.0	7.4	7.2
Calcium and Magnesium [2]						
Water extract:						
Total Ca	0.10	0.11	0.08	0.07	0.11	0.12
Ca^{++}	0.06	0.07	0.05	0.05	0.05	0.05
Total Mg	0.40	0.41	0.44	0.44	0.50	0.51
Mg^{++}	0.37	0.36	0.33	0.35	0.43	0.47
Juice:						
Ca^{++}	0.02	0.03	0.01	0.02	0.01	0.01
Mg^{++}	0.21	0.16	0.10	0.11	0.13	0.14
Moisture loss [3]						
40° C	20.3	9.8	25.9	14.8	31.4	20.6
70° C	47.1	33.5	50.2	36.6	52.6	44.2

[1] Percent total N extracted.
[2] Milliequivalent per 1 g. total N of the muscle.
[3] Percent of total moisture.

Effect of modifying meat after freeze-dehydration. Many experiments were conducted using control, 2 and 15 days post mortem aged and modified dehydrated meat samples [c] to establish: (a) the effect on the water holding capacity of adding various concentrations of NaCl, KCl, $MgCl_2$ and Na-ascorbate to the water used for rehydration and (b) the most suitable concentration of these solutes, singly or in combination which exert no objectionable effect on the acceptability of the rehydrated meat but still have a pronounced effect on the water holding properties.

The meat used for all these experiments was freeze-dried by the Qm. Food and Container Institute for the Armed Forces, Chicago. Results of these experiments indicated that rehydration of the meat samples in a solution containing 0.2 M NaCl, 0.01 M KCl and 0.05 M sodium ascorbate seems to change the texture of the rehydrated meat and increase its water holding capacity with no objectionable effect on the flavor. Adding either or both calcium and magnesium chlorides to the water of reconstitution increased the water holding properties but the rehydrated meat sample had an objectionable taste even when small concentrations of these solutes were used. It should be stated that rehydration of a previously modified meat produced more

[c] The meat was modified through the infusion of round muscles with a modifier solution containing 2.06 M NaCl, 0.27 M KCl and 0.01 M Na-ascorbate (modifier Soln/meat = 1/25) and then freeze-dried after 2 days post mortem. This ratio of modifier solution/meat kept the water holding properties of the meat at a more constant level during post mortem aging.

acceptable meat with higher water holding properties than the modification of control meat.

Effect of cooking prior to freeze-dehydration. Control and modified meat samples of 2 animals were used in these experiments. The modification was accomplished through infusion of an additive solution containing 2.06 M NaCl, 0.27 M KCl and 0.01 M Na-ascorbate using a ratio of 1:25 (modifier solution/meat). Infused rounds and their respective controls were kept at 3° C. post mortem and representative samples were obtained for analysis after two days post mortem and after cooking. Control and modified cooked meat samples were frozen, freeze-dehydrated, and canned in Chicago. Dehydrated cooked samples were reconstituted and then analyzed. Results were as follows:

Whereas cooking increased the pH of both meat and the juice released probably due to destruction of some sulfhydryl and carboxylic groups and the liberation of some basic amino groups, freeze-drying had very little effect. Heat denaturation due to cooking resulted in a marked decrease in the water and the 4% trichloroacetic acid soluble proteins. Freeze-dehydration of the cooked meat on the other hand increased the concentration of these soluble protein fractions probably due to permeability changes of the muscle fiber[4] membrane (the sarcolemma) allowing more of these soluble nitrogen components such as some peptides and nucleotides to diffuse out during the extraction procedure. Freeze-dehydration may also reverse the solubility of some denaturated protein resulting from cooking leading to the increase of the water and trichloroacetic acid soluble nitrogen. The work of Anson and Mirsky *(1, 2)* on trypsin may support this speculation. These authors stated that cooling trypsin that have been boiled (denatured) for a short time may lead to a recovery of native trypsin.

The effect of freeze-drying the cooked meat on the calcium and the magnesium in the water extract showed that both total calcium and total magnesium increased.

Freeze-dehydration of cooked meat also affected the water holding properties of both the control and modified rehydrated meat samples. This effect was more pronounced on the control cooked meat samples compared to the modified. It seems that structural changes in the denatured protein are taking place during freeze-dehydration. Modification of the meat, using this low concentration of modifiers, favorably affected the cooked meat as indicated by the high scoring of the modified meat in tenderness and juiciness compared to the control samples. Freeze-dehydration of the cooked meat exerted an unfavorable affect on both the control and modified meat samples. Both the samples developed an undesirable odor and flavor, which may be due to fat rancidity. Light green color was observed in some freeze-dried samples.

Preliminary experiments to decrease the time required for freeze-dehydration of meat. The time required for freeze-dehydration of meat, using a plate temperature of 43° C. and a chamber pressure of 1500 μ Hg., to reach a moisture content of 2-4%, was 24 hrs. When lower temperature and pressure (22-30° C. and 300-400 μ Hg.) were used for the freeze-dehydration,

[4] The muscle fiber is a fusiform cell with many nuclei.

this time increased to 28 hours and 33 hours for the control and the modified meat samples, respectively.

Experiments were conducted to decrease the time needed for freeze-drying of the meat samples using the low temperature and pressure. Aluminum trays $\frac{1}{8} \times 15 \times 30$ in. with and without aluminum nails were constructed. These nails (½-¾ in. long) were placed one inch apart and perpendicular to the plane of the trays. Control and modified meat samples were frozen on these trays, for a total period of 24 hours at —40° C., then dehydrated using a plate temperature of 22-30° C. and a chamber pressure of 300-400 μ Hg. The results of these preliminary experiments indicated that freeze-dehydration of ½-¾ in. steaks to a moisture content of 2-3% can be accomplished within 15-20 hours for the control and modified meat using both types of aluminum trays. It should be stated that the freeze-dehydrated meat using aluminum trays with nails were rehydrated more quickly and had a better texture compared to the same meat dehydrated on aluminum trays without nails. Case hardening of the meat was detected in some of the samples during the rehydration process. This was noticed when the temperature of the meat increased to 15-20° C. or more during the final stages of the dehydration process.

Thickness of the steak and modification of the meat through vascular infusion of inorganic ions appeared to be among the factors affecting the rate of freeze-dehydration.

Ballantyne *(4)* reported using aluminum trays with nails for the freeze-dehydration of meat. He stated that these nails increased heat penetration and thus reduced freeze-drying time.

Electrophoretic study of the muscle proteins. The electrophoretic properties of water soluble and buffer soluble fractions in muscle structural protein were studied in order to investigate the changes which take place during the freeze-dehydration. Veronal buffer (pH 8.6 and ionic strength = 0.1) extracts from the loin and the round muscles of fresh and freeze-dehydrated meat samples were used in this investigation.

Electrophoretic patterns representing muscle extract of loin, 2 days post mortem, before and after freeze-dehydration are shown in Figure 1. Duration of the electrophoresis and the electrical field strength were 11,000 seconds and 8.8 V/cm, respectively.

There are several protein components in the buffer extract of fresh meat. These components are of different mobilities and their identities as yet are not known. Based on the mobilities of these different protein components and comparing them with those in the available literature *(8, 9, 10, 11, 12, 13)*, it can be stated that the fast moving component appears to be myoalbumin, followed by actomyosin then myosin and finally myogen*. It should be stated that several enzymes have been reported to be present in these components *(5, 18)*. Myogen component may contain aldolase. The actomyosin and myosin group may contain phosphoglyceraldehyde dehydrogenase and phosphoglucomutase. Between these components and that of myoalbumin, phosphatases a and b have been reported as well as globulin X of Weber *(23)*.

* Myogen contains sarcoplasmic proteins and some of the muscle granules.

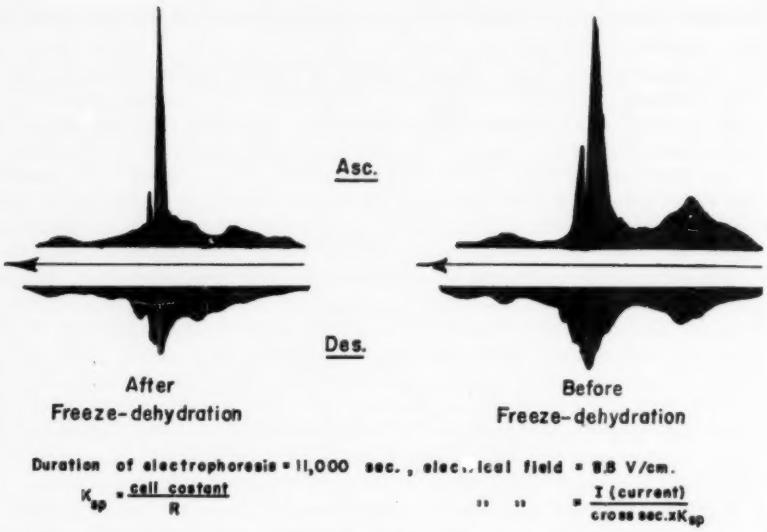

Fig. 1. Electrophoretic patterns of beef muscle extract, 2 day post-mortem at $/_2 = 0.10$ and pH 8.6.

When the meat was freeze-dehydrated it was observed that the concentration of these different components markedly decreased as indicated by the areas under the different peaks and also by determining the percentage total nitrogen extracted in the veronal buffer. Myosin seems to be greatly affected during the freeze-dehydration process.

When freeze-dehydration of meat was accomplished using lower temperature and pressure (22-30° C. and 300-400 μ Hg.) smaller changes were detected in the concentration of all these different components except myosin. It was also observed that the concentration of the myosin decreases under these conditions.

SUMMARY

Freeze-dehydration exerted no effect on the pH of both control (2 and 15 days post mortem) or modified meat. Freeze-dehydration (using 43° C. and pressure of 1500 μ Hg.) markedly affected the water and buffer soluble proteins as measured chemically and electrophoretically. Total calcium concentration in the water extract increased upon freeze-dehydration while ionic calcium decreased. The water holding properties of the rehydrated meat was independent of post mortem aging. The improvement in the water holding properties of the meat as a result of vascular infusion was not carried over to the meat samples. It seems that freeze-dehydration of the meat under this temperature and pressure profoundly and adversely affected the protein structure.

When the modified meat samples were freeze-dehydrated using lower temperature and pressure (22-30° C. and 300-400 μ Hg.) little effect on both the water and the trichloroacetic acid soluble proteins was detected. The

water holding capacity and the eating quality of the modified meat were improved.

Vascular infusion of a modifier solution containing 2.06 M NaCl, 0.27 M KCl and 0.01 M Na-ascorbate at a ratio of 1/25 (modifier solution/meat) favorably improved the water holding property of meat and maintained it during post mortem aging.

Aluminum trays with and without nails were used to decrease the time required for dehydration. Freeze-dehydration of ½-¾ in. steaks to 2-3% moisture can be accomplished using a plate temperature of 22-30° C. and a chamber pressure of 300-400 μ Hg. within 15-20 hours.

Rehydration of the freeze-dried meat in a modifier solution containing 0.2 M NaCl, 0.01 M KCl and 0.05 M Na-ascorbate modifies the physical properties of meat and increases its water holding capacity with no objectionable flavor. Rehydration of previously modified meat in this solution produced more acceptable meat both in texture and water holding properties than the modification of control meat.

Although a considerable decrease was noted in the concentration of the water and trichloroacetic acid soluble proteins as a result of cooking the meat, freeze-dehydration of the cooked meat increased the concentration of both protein fractions. Modification of meat favorably affected the water holding properties of cooked meat while freeze-dehydration adversely affected cooked meat leading to a product with a very dry and woody structure.

Acknowledgment

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COFFEE GRINDS. II. CLASSIFICATION AND ANALYSIS*

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Although it has been known for many years that roasted coffee beans must be ground before beverage can be prepared, nothing has been published concerning the particle size of material that can be produced by a wide variety of grinding equipment available for plant, store, and home use. Only one publication *(1)* has described in detail the method by which a grind can be analyzed, shown the relationship between grind composition and sieve shaking time, and mentioned precision of measurement. There is also only one publication *(2)* that attempts to classify consumer grinds, to define the particle size distribution, and to establish tolerances within which the distribution of particles for these grinds should fall. In an industry that grinds for one purpose or another approximately two and one half billion pounds of beans annually in the United States alone and whose product has a value of two billion dollars, this lack of published information about one of its major operations could become a handicap and should be remedied.

There is already in existence a vast amount of information and experience in industry that could easily be gathered together and summarized. There is nothing about the grinding operation, as practiced today, that cannot and should not be discussed freely. In many other divisions of the food industry, discussion and publication of information has furnished a reservoir of useful technical reference data. Data on the grinding operation would benefit all segments of the coffee industry and allied operations and, in particular, the consumer.

This paper will discuss grind classification and analysis and attempt to show how some of the existing shortcomings of each may be corrected.

GRIND CLASSIFICATION

In the past grinds have been classified according to many terms — steel-cut, corn meal, regular, coarse, medium, fine, percolator, drip, Silex, vacuum and pulverized — or by numbers derived from grinder settings. The classification used in the U. S. Department of Commerce recommendation (Table 1) includes only three of these, regular, drip, and fine. This is an ambiguous classification in that the first term, *regular*, has no basic meaning; and the second, *drip*, refers to a type of equipment, of which there are many varieties. The third term is the only one that relates specifically to particle size.

The terminology used in the classification of particle size should at least be homogeneous in connotation. It is not necessary to search for words to classify descriptively different degrees of subdivision in particulate material, like ground coffee. They already exist and are used to describe the state of similar materials, like sugar or sand. It is suggested that the designations regular and drip be replaced by the terms "coarse" and "medium".

These descriptive terms, together with "fine", comprise a basic grading scale of 3 terms. It may be extended naturally to include a number of other terms, each of which will convey a very definite meaning. For example, a 5-point grading scale might contain the terms, very coarse, coarse, medium, fine and very fine. Further extension to

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TABLE 1

Recommended grinds for coffee [1]

Grind designation	Amount of coffee retained on		Amount of coffee passing through control sieve 28-mesh	Tolerances on amount of coffee passing through control sieve 28-mesh	
	10 and 14 mesh sieves	20 and 28 mesh sieves		Not less than	Not more than
	%	%	%	%	%
Regular	33	55	12	9	15
Drip	7	73	20	16	24
Fine	0	70	30	25	40

[1] U. S. Dept. Commerce R 231-48

TABLE 2

Terminology for the grading of coffee grinds

Three point scale	Seven point scale	Nine point scale	Eleven point scale
	extremely coarse	extremely coarse	extremely coarse
	very coarse	very coarse	very coarse
Coarse	coarse	coarse	coarse
			coarse — medium
		medium — coarse	medium — coarse
Medium	medium	medium	medium
		medium — fine	medium — fine
			fine — medium
Fine	fine	fine	fine
	very fine	very fine	very fine
	extremely fine	extremely fine	extremely fine

7-point, 9-point and 11-point grading scales is shown in Table 2. How these grading scale terms relate to particle size and percentage fractions in a common commercial grind will be shown below. Their association with the names used to differentiate the grinds found in the grocery trade will also be described.

The ground material leaving a grinder is not homogeneous with respect to size. The particles in grinds prepared for use in home brewing equipment vary in size, depending upon the distance between cutting rollers or plates, from about 0.09 in. to less than 0.01 in. in average diameter. The particles are irregular in shape and their average diameter determined by the size of screen openings through which they will and will not pass. The selection of screens for particle size measurement is quite arbitrary and based on the range of sizes generally encountered in commercial practice.

There has been no attempt to formalize the grind analysis data. The U. S. Department of Commerce recommendation (Table 1) makes a differentiation among the 3 grinds listed according to the proportion of material passing through the number 28 (Tyler) screen (pan fraction). For these the middle values and tolerances for each grind are: regular, 12 plus or minus 3%; drip, 20 plus or minus 4% and fine, 30 plus 10 or minus 5%.

The method, reported by the author in April 1958 *(1)*, if skillfully applied and carefully controlled, will distinguish between grinds whose pan fractions differ by as little as 0.7%. If for convenience, the difference limit is taken as one percent, then 100 gradations of grind are theoretically possible. This is useful because any grind whose pan fraction differs from that of other grinds by at least 1% can be called a different grind. Furthermore, the percent scale without subdivision or contraction will have practical meaning. This will also permit a direct combination of the 11-point descriptive scale with a numerical scale based on percentage and limits for each class (Table 3).

This system now allows a more reasonable interpretation of grind analysis than that proposed by the earlier (USDC) recommendation. It establishes (a) the terminal points on the numerical scale, zero and 100%, (b) the unit of scale subdivision, 1%, (c) the limits within which a descriptive term applies, 5 - 15, 15 - 25, etc., (d) the mid-point for each class to which a descriptive term has been attached, 10, 20, 30, etc., (e) a uni-

TABLE 3
Correlation of descriptive and numerical scale

Common name	Eleven point descriptive scale	100 point percentage scale [1] (mid-points)	Class limits
	extremely coarse	0	0 – 5
Percolator	very coarse	10	5 – 15
Drip	coarse	20	15 – 25
Vacuum	coarse — medium	30	25 – 35
	medium — coarse	40	35 – 45
	medium	50	45 – 55
	medium — fine	60	55 – 65
	fine — medium	70	65 – 75
	fine	80	75 – 85
	very fine	90	85 – 95
	extremely fine	100	95 – 100

[1] Based on percentage of sample passing No. 28 screen.

form tolerance for each class, plus or minus 5, (f) a natural descriptive terminology, extremely coarse, very coarse, coarse, etc. and (g) a place for any grind that may be produced by the coffee industry.

In contrast, the earlier (USDC) recommendation cannot provide for other grinds which are commercially practical; its class limits are not uniform; its tolerances for each class are not uniform and not continuous, for example, in which class would a grind having a pan fraction value of 15.5 or 24.5% fall? As pointed out, its descriptive terms are ambiguous and do not comprise a family of terms having closely associated connotations.

For the convenience of the consumers, common designations for grinds need not be abandoned. The regular grind which is used in percolators is also called, as one might expect, a percolator grind. Its designation as a percolator grind should be continued, although according to the proposed classification and among coffee technologists, it would be called a very coarse grind. By the same reasoning, the terms drip and vacuum for grinds which are used in home brewing devices differentiated by those names, are still appropriate. They would, however, be classified as coarse and coarse-medium grinds respectively by the technologists.

GRIND ANALYSIS

Typical data obtained by using the method recommended by the U. S. Department of Commerce *(2)* and described in detail *(1)* are shown in Table 4. Both of these samples contain about the same amount of fine material; the pan fractions are 19.9 and 22.0%. According to both the recommendation (Table 1) and the proposed system of classification (Table 3) these grinds are drip or coarse grinds. However, closer inspection of the data not used for the purposes of classification reveals that only one of these samples, sample B, has fractions retained on the several sieves conforming closely to those required by the recommendation (Table 1). The non-conformity in sample A can only be attributed to atypical or worn grinding equipment or to improper adjustment of the grinding mechanism. Still, according to the recommendation, both grinds would have to be classified as drip grinds because the pan fraction values which is the controlling fraction, are very close to 20%.

This interpretation of actual data clearly points out the need for an examination of all of the data obtained from the analysis in order to arrive at a clear picture of the distribution of particle sizes in a grind.

TABLE 4
Analysis of coffee grinds

Portion held by	Sample A			Sample B		
	1	2	Avg.	1	2	Avg.
	%	%	%	%	%	%
10-mesh	3.0	3.0	3.0	0.1	0.3	0.2
14	25.8	25.8	25.8	5.5	5.3	5.4
20	35.7	35.7	35.7	37.9	37.0	37.4
28	15.6	15.8	15.7	35.2	35.0	35.1
Pan	19.8	19.9	19.9	21.8	22.3	22.0

Summary (according to the Standard Practice Recommendation)

	Sample A	Sample B
Percent held by		
10 and 14 mesh	28.8	5.8
10 and 28 mesh	51.4	72.5
pan	19.9	22.0

Ordinarily, a grind analysis report contains only 3 values: one, the fractions retained by both the number 10 and 14 screens; two, the fractions retained by both the number 20 and 28 screens; and three, the fraction passing through the number 28 screen into the collecting pan. The reason for combining data in this manner is not clear and has never been justified. In fact, in doing so, some of the original information required for a closer examination of particle size distribution is lost. Consequently, this practice should be discontinued.

Data from a screen analysis can be plotted on logarithmic probability paper (Figure 1). When this is done, an excellent view of grind characteristics and interrelationships appears. In this graphical presentation, ordinates are particle sizes in hundredths of an inch and for convenience the size of openings in screens throughout the range from number 9 to 48 in the Tyler series are also given. Abscissas show percentages of the sample retained on any screen or on any combination of screens. If only the alternate screens, numbers 10, 14, 20 and 28, are used, there is enough data for a useful plot

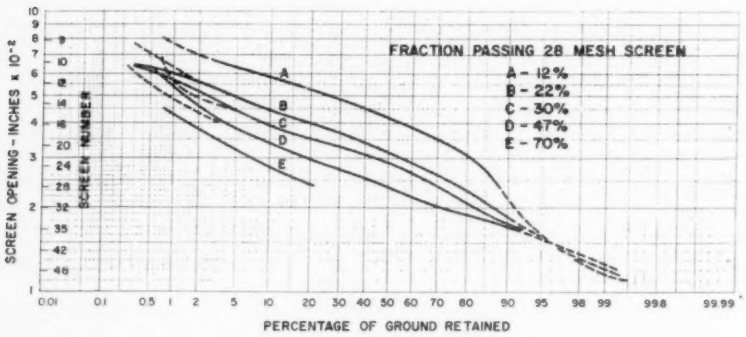

Fig. 1. Analysis of coffee grinds.

in most cases. However, if finely ground samples are analyzed, so much material passes through the number 28 screen, that the coarser screens retain nothing or very little. In this case, curves cannot be plotted. To analyze a so-called fine grind or those that are more finely ground, it is necessary to replace coarse screens with several others having openings smaller than that of the number 28 screen. It has been useful, on occasion, to run grind analysis with a full complement of 12 screens ranging in number from 9 to 48. When the 4 screens mentioned above are used, the size differential is a factor of the square root of two, or 1.414, and the size range covered is 0.0232 to 0.0661 inches. When the full set of screens is used, the size differential is a factor of the fourth root of two, or 1.189, and the size range covered is 0.0117 to 0.0789 inches.

The curves A, B and C, are representative of the 3 basic commercial grinds. Curve D, which has a fine fraction equal to 47% was prepared with data from a commercial coffee. According to the proposed terminology, this would be called a medium grind. Curve E portrays a fine-medium grind which can be obtained from a home grinder. The solid lines are derived from 4-screen analysis; the dashed lines are derived from 12-screen analysis. The two finer grinds cannot be usefully described by a 4-screen analysis. Data from further fractionation with finer screens are necessary to characterize them properly.

From a graphical presentation of grind data, information about particle size distribution can be readily derived. If curve A is taken as an example, the mean size of particles in the sample is 0.041 inches. This value is obtained by reading up from the 50% point on the horizontal scale to the curve and then over to the size scale on the left. The standard deviation, an important characteristic of any distribution and a measure of the particle size dispersion about the mean, can be derived in the same way as the mean by reading up from the 15.9 percentage point on the horizontal scale to the curve, then over to the size scale on the left and then subtracting from this value that of the mean. For sample A the standard deviation is 0.013 (0.054-0.041) inches. The size value obtained by operating in a similar manner from the 84.1 percentage point is 0.014 (0.041-0.027) inches.

As these two values, representing plus and minus one standard deviation from the mean are quite close, it may be concluded that at least 68.2% of the particle sizes in the sample are normally distributed about the mean.

If size values corresponding to the 2.3 and 97.7 percentage points are found, the differences between these and the mean should also be equal. These differences are 0.027 and 0.028 inches respectively; each is twice the values found for plus and minus one standard deviation. As these values, representing plus and minus two standard deviations from the mean are quite close, it may be concluded that at least 95.5% of the particle sizes in the sample are normally distributed about the mean. It may also be concluded that the grinding operation is producing a normal grind.

If this type of analysis is applied to curves B, C and D, one finds that the mean values decrease, as might be expected. However, while the distribution of particle sizes larger than the means is normal, that of those sizes smaller than the means becomes somewhat distorted. There is a larger proportion of smaller particles than expected.

Further analysis shows that as the particle sizes become smaller, the standard deviations associated with each sample become smaller. However, the range of sizes included by about 95% of each sample is so great that none among the group, represented by curves A, B and C, are statistically different. In fact, large proportions of each sample are identical in particle size. This observation derived from a study of grind analysis favors the proposed classification which provides a more rational picture of basic commercial grinds and emphasizes their true similarity.

Implications. The implications of this preliminary analysis of coffee grinds are as follows: (1) because of the great similarity among the 3 commercial grinds which account for more than 60% of all ground coffee, the grinding operation should be re-examined to determine whether 3 grinds are actually required or whether only a single grind chosen from these three would suffice; (2) if different grinds are actually needed for the proper performance of the 3 kinds of brewing equipment currently used in the home, then a greater physical differentiation among the grinds should be achieved by screening grinder product or by new grinder design; (3) the proportion of extremely fine particles in a grind should be reduced by the same approaches; (4) other commercial grinds should be carefully analyzed to determine more adequately their characteristics and differences not only basic but also with respect to intended use; (5) a closer working arrangement between coffee roasters and equipment manufacturers for a more intimate understanding of mutual problems should be developed; (6) only those grinds and brewing equipment which will yield beverage having maximum aroma and optimum taste should be produced; and (7) more data on grinds are needed and an application of modern statistical analysis is necessary for a useful interpretation of their meaning.

SUMMARY

The present system of classification of coffee grinds has been examined and its inadequacy and ambiguities pointed out. A more natural and rational system is proposed. A simple statistical approach to grind analysis and grind characterization reveals that the three grinds representing the largest proportion of ground coffee are quite similar. Implications derived from this fact are outlined. The need for more information about grinds is emphasized.

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ON THE FEASIBILITY OF ULTRASONIC GRADING OF SHELL EGGS[a]

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Investigations described in this report were undertaken to determine whether or not it is feasible to develop and evaluate ultrasonic methods for measuring albumen quality of shell eggs and to relate these qualities to the grade of the egg. In the course of the study it became apparent that the geometry of the shell influences the measurements significantly. This paper reports on the systematic measurements of ultrasonic velocities and absorptions in the various parts of the shell egg and on the relationships between these measurements and the possible grading of the eggs.

EXPERIMENTAL METHODS

The electronic equipment used in the experiments was constructed in such a manner that both velocity and absorption measurements could be made.

A block diagram showing the arrangement of the electronic apparatus is given in Figure 1. The pulsing circuit is activated by a steep electronic pulse of 1 microsecond

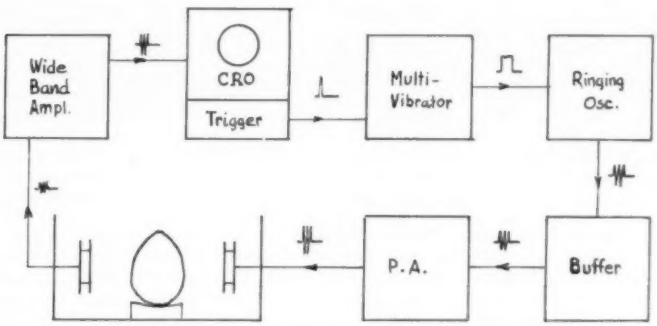

Figure 1. Block diagram of electronic apparatus.

duration which is produced by a range oscilloscope at the same time the sweep of the oscilloscope is started. This pulse is used to activate a single-shot multivibrator whose output can be varied to control the length of the ultrasonic pulse. The output of the multivibrator is fed into a ringing oscillator which controls the modulation frequency of the final pulse. After suitable amplification the signal is applied to a transducer to convert the electrical energy to ultrasonic energy. This energy is now available in the form of ultrasonic pulses whose amplitude, modulation frequency, repetition rate, and duration can be varied at will.

Velocity and absorption measurements are generally made in the following manner. The ultrasonic pulse leaves the transducer at the same instant the sweep of the oscilloscope is started, enters the sample, travels through the sample with a certain velocity and reaches the other side of the sample after a certain time where the signal is picked

[a] Report of a study made with funds from the Research and Marketing Act of 1946.

up by another transducer. The received signal is amplified and displayed on the screen of the oscilloscope. While the ultrasonic pulse travels through the sample the sweep of the oscilloscope advances across the screen, visible as a straight line, until the pulse reaches the receiving transducer whose output deflects the sweep vertically. A calibrated time scale of the oscilloscope is used to determine the time elapsed between the departure of the ultrasonic pulse from the transmitting transducer and its arrival at the receiving transducer. From the knowledge of the dimensions of the sample and the time difference, the velocity in the sample is calculated.

The losses the ultrasonic pulse experiences in the sample are found by a comparison of signal amplitudes with the sample in the sound path and without the sample. Essentially, this is done by observing the signal amplitude with the sample in the path and then removing the sample. This removal causes the signal amplitude to increase. This increase is measured by inserting in the receiving circuit attenuation elements until the signal is reduced to the exact amplitude it had with the sample in the path. This artificial reduction corresponds to the losses introduced by the presence of the sample, consisting of absorption in the specimen and the possible decrease in ultrasonic intensity due to the difference in the acoustic impedance between the surrounding medium and the substance under investigation.

Before it was possible to correlate the velocity and absorption measurements to the grade or quality of the shell egg, it was found advisable to investigate the velocities and absorptions in the different parts of the egg.

The Poultry Department, Michigan State University, supplied the eggs needed for the experiments as well as 2 samples of albumen collected from a number of A quality eggs. The quality was determined by break-out tests. To find the ultrasonic velocity in albumen as a function of temperature and age, the 2 samples were investigated with the pulse method.

RESULTS

The ultrasonic velocity in thick albumen is almost the same as that in thin albumen when the samples are fresh; the temperature dependence varies extremely little for both samples. Thin albumen shows a greater change in ultrasonic velocity due to aging than thick albumen. This can be attributed to the greater change in viscosity of thin albumen; thick albumen does not seem to change its viscosity as rapidly. In all cases the velocity increases with an increase in temperature. The numerical values for thin albumen can be obtained from Figure 2. After the 5th day the thickness of the albumen had increased quite markedly, and its volume had decreased. The volume lost by evaporation was replaced by adding water to the sample. The ultrasonic velocity was lower than the original velocity in the fresh sample. This would indicate that not only evap-

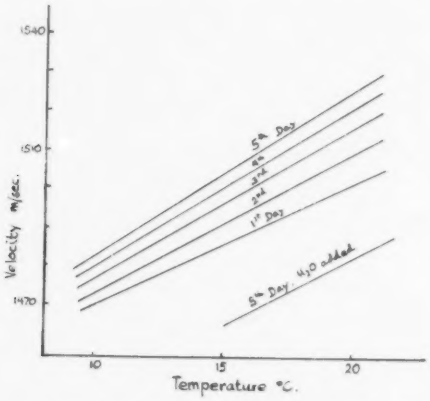

Figure 2. Ultrasonic velocity in albumen as function of temperature and time.

oration had taken place but also that some structural change of the albumen had occurred. The change in ultrasonic velocity does not indicate what changes have taken place. Thick albumen shows essentially the same behavior u der similar conditions.

Absorption measurements in a fairly large sample of albumen yield only average values. The presence of lumps of albumen with a viscosity higher than that of the rest of the sample will tend to influence the absorption readings, and since some of the ultrasonic intensity may be scattered by the high viscosity albumen, one can only say that a recorded absorption reading indicates a maximum value of attenuation. In the case of the samples examined this absorption is not more than 0.3 db/cm.

The foregoing statements refer to a mixture of albumen from a number of eggs. To see whether the velocities in the yolk and the albumen differ very much from egg to egg, a small vessel was constructed, equipped with a transducer on the bottom and a plunger which can be raised and lowered in the vessel. A transducer is cemented to the plunger, and the distance between the 2 transducers can be determined accurately to within 0.01 mm. The space between the transducers in the vessel is filled with albumen or yolk from one egg alone. Moving the plunger through a certain distance, and noting the corresponding change in travel time of the ultrasonic pulse, gives the velocity in the sample.

Most of the velocities in A quality Large eggs fell within the following limits:

Yolk: 1510 m/sec — 1570 m/sec.

Albumen: 1505 m/sec — 1565 m/sec.

The albumen and the yolk used in these measurements were taken from eggs evaluated by break-out tests. Variations in differences between the velocities in the yolk and the albumen of the same egg were found to be in the following limits:

Yolk velocity 19 m/sec smaller than albumen velocity.

Yolk velocity 12 m/sec greater than albumen velocity.

All these measurements were made at 24° C.

Similar measurements were taken for eggs of lower quality. The results follow the same pattern, and a differentiation between high and low quality shell eggs based on the results of velocity measurements in the yolk and albumen alone seems to be impossible since the results of the individual measurements vary not only within one group of eggs but also overlap between the groups. No trend, either higher or lower velocities, could be detected which might be helpful in identifying an egg as belonging to A quality or to a lower grade.

In the course of these investigations it was found that the transmission of ultrasonic energy through a whole egg depends on the frequency of the ultrasonic beam or pulse as well as on the direction of propagation with respect to the geometry of the shell egg. The absorption in the intact egg was found to be much greater than expected from absorption measurements in the yolk and albumen alone. A systematic study of the transmission properties of the shell was therefore undertaken.

In a homogeneous medium the absorption of ultrasonic energy is a function of the frequency: the higher the frequency, the higher the losses. For a non-homogeneous medium, one has to consider the size and the form of the irregularities. An egg shell must be treated as a non-uniform medium on account of its porous character where the size of the pores determines the maximum frequency of the ultrasonic beam which can still be transmitted. In order to obtain meaningful readings one must use an ultrasonic beam whose cross section remains constant throughout its path. The amount of spreading of the beam as it travels away from the transducer is a function of the diameter of the transducer and the wave length in the medium through which the energy travels. If the medium in which the egg is immersed is water or mineral oil (velocity approximately 1500 m/sec), a uni-directional beam or pulse at 1 Mc can be obtained if the diameter of the radiating face of the transducer is about the same as the width of the egg.

If one now uses such a wide 1 Mc beam it is obvious that some of the emitted energy will bypass the egg completely. Some energy will be reflected from the shell without even entering the egg, and only a very small fraction will enter the egg. From this it can be seen that at low frequencies and wide beams, measurements become unreliable due to external and internal reflections while at frequencies above 5 Mc the losses due to the porous shell become rather high. The above considerations are valid whether the ultrasonic velocity through the egg is to be measured or the absorption in the egg is to be

observed. In both cases one has to know through which parts of the egg the beam or pulse travels after it leaves the shell on the inside of the egg. In this case one has to use the theory of sound transmission through thin plates (the egg shell). Let one plane wave front approach the egg shell from the outside. This wave front will strike the shell at many different angles, i.e., the angle between the wave front and the normals to the egg shell will change from place to place due to the curvature of the shell. Experiment and theory show that the amount of transmission through the plate is a function of this angle. At a certain angle, in the case of mineral oil and egg shell this angle is about 30°, the transmission is cut off completely which means that the sections of the wave front striking the shell at an angle greater than 30° will be totally reflected and will be lost for the purpose of the measurements. Thus it becomes impossible to transmit the signal through the long dimensions of the egg since the curvature at the top and the bottom of the egg changes quite rapidly, and there exists only a relatively small area of the shell where the angle between the normal to the shell and the direction of sound propagation is less than 30°. In addition, most of this area is unsuited for transmission of ultrasonic energy into the egg since the air cell in the egg occupies the space between the shell and the rest of the egg at the area where sound could enter. Since there is very high impedance mismatch between air and solid matter it is practically impossible to transmit ultrasonic energy through the air cell. It is for this reason that the egg must be irradiated from the side despite the fact that this means a shorter path length through the sample with an associated increase in the uncertainty of the measurements. In order to receive behind the egg a reasonably plane wave front one should use a relatively narrow ultrasonic beam to minimize the effects of the different curvatures of the shell and other layers inside the egg.

The effects of a curved wave front on the shape of the signal can be studied by using 2 transducers, activating one with short (about 20 microseconds) electric pulses, and using the other to receive the ultrasonic pulses after they have traveled through the egg. It was found that the shape of the received signal is much more distorted than would be expected. An ultrasonic pulse consisting of about 50 waves, does not cross the shell in such a manner that every part of the pulse is delayed in the shell by the same amount of time. It is important to know this amount of delay if one is to use velocity measurements. The reasons for the irregular delay times of the sections of the pulse are known. A theoretical analysis was made by Schoch *(1)* for plane thin plates but a numerical evaluation becomes rather complex and does not lead to results which can be used in the solution of the present problem.

The effects of the distortions on the pulse after 2 transmissions of the shell (into the egg and out again) is shown in Figure 3 which was taken from a photograph and drawn so that the amplitudes of the signal appear to be equal. The amplitude of the pulse through the egg is actually much lower. Inside the egg the sound pulse must travel across layers of albumen and yolk. The boundaries between these sections present curvatures to the pulse which are different from the curvature of the shell. Since the acoustic

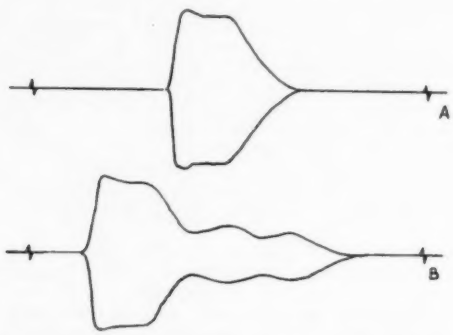

Figure 3. Oscillogram of ultrasonic pulse. A. Undistorted form without egg. B. Distorted form with egg in path.

impedances of yolk and albumen differ by very little it is doubtful whether the boundaries between them influence the transmission a great deal. Nevertheless, the propagation direction of the spatial sections of the pulse may be changed somewhat. The pulse can experience an additional distortion due to the different path lengths of different sections of the pulse. It is extremely difficult to separate the distortions of the pulse due to the path differences from those due to the delay in the shell.

In trying to use the absorption properties of the egg for grading purposes, one encounters the following difficulties. It was mentioned before that it is important to irradiate an area of the shell so that a minimum change of curvature occurs over the area. It was pointed out that this condition can be met if the pulse impinges at about the mid-section of the egg. It was found that the absorption in the shell varies with the individual egg. Furthermore, the absorption is extremely high, on the average about 20 db/cm. The attenuation due to the shell is therefore much greater than the absorption in the region of interest, namely the yolk and the albumen (about 0.3 db/cm). Consequently, it is difficult to detect the the absorption due to the inside of the egg. It was found that a slight displacement of the entire egg normal to the direction of ultrasonic propagation introduces a change in absorption many times greater than the total absorption in the yolk and the albumen. For consistent measurements it is therefore important to expose the shell perpendicularly to the beam in such a manner that the opposite side of the shell is also perpendicular to the direction of propagation. Figure 4 illustrates why the absorption will appear to be higher if only one side of the egg is at right angles to the ultrasonic beam or pulse.

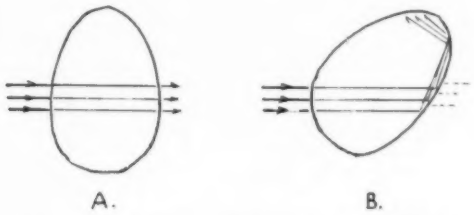

Figure 4. Positions of egg in ultrasonic beam for absorption measurements. A. Correct position. Minimum losses by reflection. B. Incorrect position. High losses.

A series of measurements of grade A Large shell eggs was made in which all the above mentioned sources of error were eliminated as much as possible. The frequency used in all cases was 2.6 Mc. Simultaneous measurements of absorption through the whole egg and measurements of the time difference with and without the egg in the ultrasonic path were recorded. It was impossible to find any meaningful correlation between the measured quantities and the dimensions of the eggs. The eggs used in these and the following measurements were first graded by candling and after the measurements by breakout tests. This can be seen from the following table which lists some typical readings.

The absorption measurements are very sensitive to the curvature of the shell and yield values greater than expected from measurements in yolk, albumen, and shell alone. The thickness of the shell does not appear to affect the results greatly. The velocity

TABLE 1
Grade A large

Time diff. (microsec.)	Width of egg (cm.)	Total attenuation (db)	Shell thickness (mm.)
.36	4.08	42	.367
1.54	4.33	30	.395
.61	4.29	46	.440
1.60	4.18	32	.412
1.57	4.25	38	.394

measurements are influenced to a great extent by the geometry of the shell and the multiple reflections in it, as well as the different delay times of the various sections of the pulse in the shell and possibly across the boundaries between the shell, albumen, and yolk.

For comparison a number of grade B shell eggs were subjected to the same tests, and the results indicate that there is no significant difference between quality A and B quality eggs as far as an ultrasonic investigation is concerned. Some representative values of the measurements are given in the following table.

TABLE 2
Grade B

Time diff. (microsec.)	Width of egg (cm.)	Total attenuation (db)	Shell thickness (mm)
1.18	4.30	48	.414
.66	4.50	40	.451
3.35	4.48	32	.388
.82	4.49	35	.385
7.59	4.38	47	.392

Comparing the 2 tables shows that there is no trend in either the absorption values nor in the time differences which could be useful for grading purposes. Time differences are listed rather than velocities. To determine the velocity one has to know the distance through which the signal travels. An actual determination of the path length is not possible since the signal experiences multiple reflections at least in the shell. This is borne out experimentally by tilting the whole egg and thus changing the angle of incidence; the absorption measurements are changed by approximately 5% while the time differences very significantly, in some cases by as much as 6 microseconds. The time differences for B quality eggs are, in general, greater than those for A quality eggs. Since the values for B quality include the range of values for A quality no definite statements can be made concerning the quality to which the egg belongs.

DISCUSSION

From results of the ultrasonic methods it is seen that the velocity and absorption in albumen from different shell eggs of the same quality occupy a considerable range of values. The values are overlapping or about equal; these measurements alone do not furnish a basis for the ultrasonic grading of eggs since the absorption of mechanical energy in yolk and albumen is found to show no dependence on the quality of the sample. At a frequency of 2.6 Mc the absorption of mechanical energy lies in a range of rather small values which is not measurably dependent on the grade of the sample.

The echo-ranging principles cannot be used to detect layers of albumen and yolk within the egg because the difference between the acoustic impedances at these layers is so small that no observable reflection of ultrasonic pulses can be obtained from the boundaries between the layers. The difference in acoustic impedance between the shell and a liquid — albumen included — is so great that echos caused by the very strong reflections at the boundaries shell and liquid are observed.

The shell is found to influence all measurements sufficiently to mask the possible differences in readings due to the qualities of the egg without the shell. Its relation to the quality was investigated but no correlation can be detected. The geometry of the shell plays a very important role in the ultrasonic absorption measurements but the structural aspects cannot be used for consistent determinations of the quality. The influence of the geometry of the shell on

absorption measurements reveals itself by the difference in "total attenuation" by a shell egg and the sum of the individual attenuations in the shell, albumen, and yolk. The total attenuation is higher than the sum since a significant part of the available ultrasonic energy is lost by reflection from various sections of the shell.

SUMMARY

The fluctuations of the velocity and the absorption of ultrasonic waves in eggs of different quality is so large that measurements of these quantities will not allow a reliable grading of eggs. Echo-ranging techniques cannot be used for determining the structure inside the shell egg, because all echos from interior boundaries are masked by the strong echos produced at the shell.

LITERATURE CITED

1. SCHOCH, A. Der Schalldurchgang durch Platten. *Acustica*, 2, 1 (1952).

MICROWAVES AS A MEANS OF GRADING EGGS[a]

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(Manuscript received May 27, 1958)

An electronic method to determine the albumen *or* interior quality of shell eggs could greatly facilitate egg marketing. Such a method might be incorporated into a machine which would automatically select eggs of the same quality. Prerequisite to any such electronic method, however, is a definite and reliable correlation between the quality of an egg and a measurable physical quantity of the egg. The conductivity at radio frequencies has been proposed as a criterion for determining egg quality *(2)*. K. H. Norris and A. W. Brant *(5)* described a method for measuring the radio frequency conductivity of highly conducting liquids over a frequency range of 50 Kc to 1000 mc. The conductivity of both albumen and yolk material from high quality eggs was found to be about 15% lower than the respective values for low-quality eggs. As this difference is rather small, it is not astonishing that a test of two electronic candlers using radio frequency gave unsatisfactory results. Norris and Brant concluded that with the equipment available at the time of their study *(5)* the interior quality of shell eggs could not be correctly measured with radio frequency.

The study reported here was made to find a more reliable quality indication by using microwave techniques. It is known *(6)* that the internal structure of the egg indicates its quality to a certain extent. As the Fresnel diffraction pattern is very sensitive to the spatial distribution of the dielectric constant inside an egg, it seemed possible to correlate changes in this pattern to the quality of the egg.

Experimental Procedure for the Diffraction Method

If microwaves are passed through an object, a diffraction pattern results. Close to the object (Fresnel zone) this pattern contains a great deal of structure and this structure is relatively sensitive to the shape and structure of the diffracting object. An attempt was made to empirically find whether differences in the diffraction pattern could be correlated with differences in egg quality and could therefore be used for the grading of eggs. The instrumentation used for this is found in Figure 1.

A 723A/B klystron is used to generate microwaves of 7.2 cm. from a circular plate. Fairly uniform microwaves pass through the egg to the receiving horn. (The dimensions of such a horn which has the desired directivity is shown in Fig. 2.) The waves are then detected, amplified and recorded in the usual manner. By automatically rotating the detector in the horizontal plane, the diffraction structure is thus automatically recorded.

EXPERIMENTAL RESULTS

It was found empirically that the most favorable configuration is that shown in Figure 1 with the egg oriented as is shown in Figure 3. In this orientation, more symmetrical patterns for A quality eggs were observed, whereas eggs of low quality generally gave unsymmetrical patterns or patterns with low peaks at the center. Three typical

[a] Report of a study made with funds from the Research and Marketing Act of 1946.
[b] Present address: University of Chicago, Chicago, Illinois.

examples are shown in Figure 4 a,b,c. The asymmetry defined as the difference, d, of the inner dips (Fig. 4c) has been plotted for each egg (Fig. 5). The experimental points along the same vertical line represent the same egg tested on 6 successive days. It is noted that some points coincide.

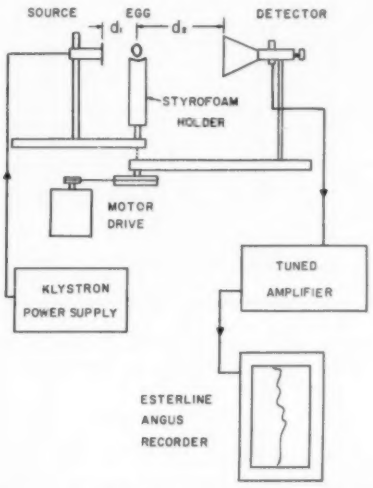

Figure 1. Block diagram of the experimental setup for the diffraction method. Configuration of source, egg and detector ($d_1 = 8$ cm, $d_2 = 45$ cm)

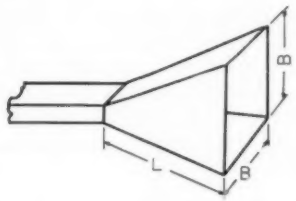

Figure 2. Dimensions of the receiving horn (L = 18.4 cm, B = 13.8 cm)

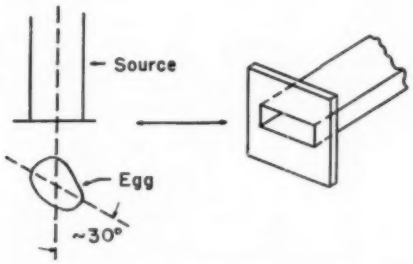

Figure 3. Orientation of egg in the diffraction method.

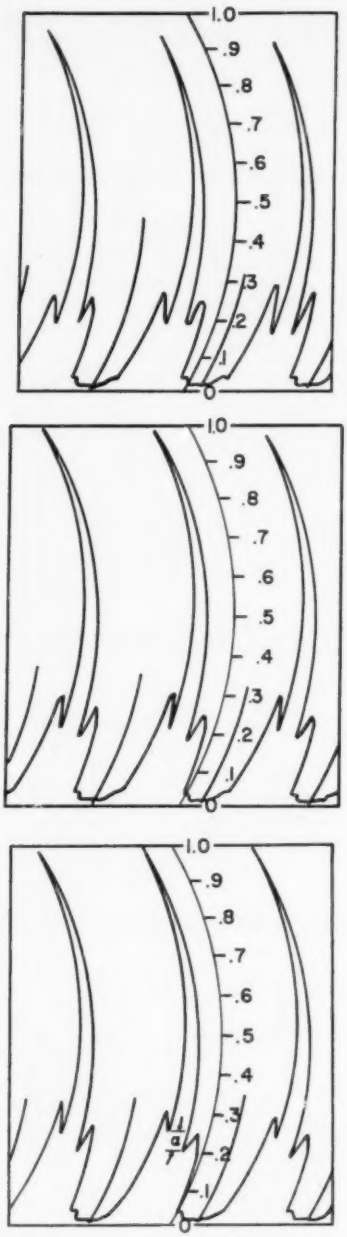

Figure 4. Diffraction patterns for eggs of: (a) A quality albumen, (b) B quality albumen, (c) C quality albumen.

It appeared possible that the frequency used was near a dielectric absorption peak caused by some special relaxation phenomenon. The 723A/B klystron was, therefore, replaced by a 2K33B klystron which can be adjusted in the range of 22,000 to 25,000 mc/s. In order to eliminate the effect of the egg shell, a teflon cylinder filled with yolk and albumen was used instead of the egg. With the teflon cylinder on the styrofoam holder and the detector being directed toward the source, the output on the scale of the audio amplifier was observed. Results proved that there is no absorption peak in this range of frequency[6]. Also there was no apparent difference in the absorption of eggs of different quality.

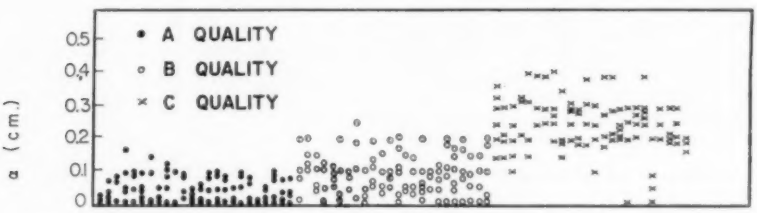

Figure 5. The asymmetry of the diffraction patterns. Points in the same vertical line represent the same egg tested on successive days. Some points coincide.

CONCLUSIONS

A study of the plot (Fig. 5) leads to the conclusion that although the diffraction method generally indicates some difference in quality, it is still not sensitive enough. Furthermore, the lack of exact reproducibility impedes any reliable conclusion. This disadvantage may be due to the shape of the shell, which cannot be controlled, or due to the difficulty in adjusting by hand and observation, the position of the egg exactly each time. Considering the outcome of the absorption test, the results indicate that the diffraction effects observed in the experiments were essentially dependent on the internal geometry of the egg. The special orientation of the egg as shown in Fig. 3 seems to suggest that the air cell near the large end may be the most important determining factor. Anyway, the geometrical difference between eggs of different quality appears not to be great enough to allow reliable grading.

INTERFEROMETER METHOD

The second method used was an attempt to observe a change in dielectric constant by use of a Fabry-Perot interferometer. A detailed theoretical development of a Fabry-Perot interferometer can be found in a paper presented by Culshaw *(3)*. A schematic diagram of the simplified form used in this experiment is shown in Figure 6. It consists of the microwave source, reflectors and the detector. The source and detector are the same as described in the diffraction method. Each reflector is constructed with four quarter-wave sheets of polystyrene separated from each other by a quarter-wave layer of air. Figure 7 indicates its structure and dimension. One important characteristic of this structure is its high reflectivity for electromagnetic radiation.

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[6] The absorption peak for the usual (Debye) type of dielectric relaxation *(4)* should be found at frequencies of the order of magnitude of 10 Kc. This frequency range is computed from values found in the literature *(4)* for the viscosity of the albumen and for the radius of its molecule. Although the measurements of electrical conductivity in this frequency were found to be unsuitable for grading, the study of dielectric absorption might lead to a reliable method.

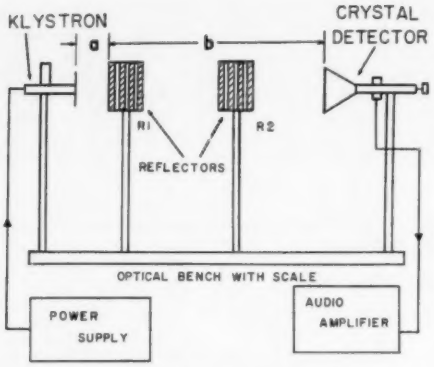

Figure 6. Schematic diagram of the interferometer (a = 12 cm, b = 46 cm)

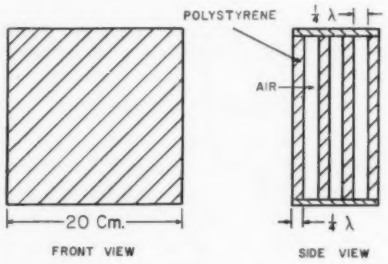

Figure 7. Structure and dimensions of the reflector.

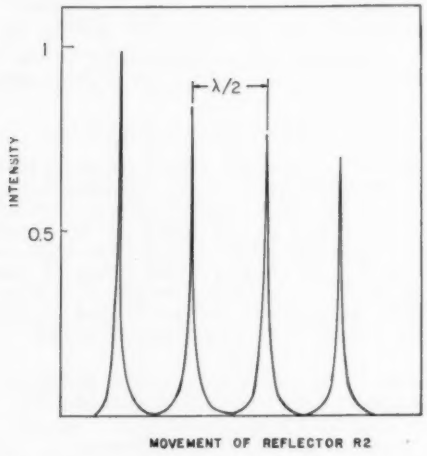

Figure 8. Sharpness of fringes on the interferometer in relation to the wavelength.

Fundamentally, the operation of this interferometer is the same as that of the Fabry-Perot interferometer in the optical region. Because of multiple reflection between the reflectors with high reflectivity, interference fringes can be observed for the transmitted radiation. However, since our interferometer operates with an essentially parallel beam, the circular fringe system of the optical model is not obtained; instead, the fringe is observed by altering the distance between the reflectors. For convenience, the reflector R1 (Fig. 6) is fixed and the reflector separation is changed by moving R2. A system of fringes obtained in this way is shown in Figure 8. The configuration of source, reflectors and detector is indicated in Figure 6. The transmitted intensity is observed on the scale of the audio-amplifier of the detecting system. It is noted that the distance between the fringes is a half wavelength as predicted theoretically *(3)*.

By studying the shift of fringes when an unknown dielectric is inserted, and comparing it to the shift of a known dielectric, the unknown dielectric constant may be determined. The simple arrangement used is a rather convenient method for *comparing* dielectric constants. It was hoped that by comparing dielectric constants of eggs of different quality, one could find a difference which could be used in an egg grading operation. A more technical and detailed account of the experimental methods used may be found in the thesis of Yen Fu Bow *(1)*.

EXPERIMENTAL RESULTS

In order to find the order of magnitude of the dielectric difference between eggs of different quality, the fringe shifts due to 3 kinds of albumen have been determined. Each kind represented one standard of quality. The fringe shifts were obtained as previously described. A cell made of polystyrene, with dimensions of 18 x 18 x 2.5 cm. and a capacity of about 500 cc, was first inserted between the reflectors and a fringe located by adjusting the reflector R2. The material under test was then put in the cell and the fringe shift observed. The results are presented in Table 1. From the mean fringe shift and the inner thickness of the cell (1.57 cm.), the relative dielectric constants can be calculated. Practically, there is no dielectric difference between albumen of different quality. The absolute dielectric constant of each kind of albumen can be estimated from the relative dielectric constant of polystyrene or teflon (Table 2) and is found to be

TABLE 1

Fringe shifts due to albumen of different quality

(Distance in cm. from R1)	Thick albumen	Less thick albumen	Thin albumen
17.0	0.90	0.95	1.00
16.7	1.13	1.15	1.15
16.4	1.02	1.02	1.02
16.0	0.85	0.87	0.87
15.7	0.80	0.80	0.80
15.4	0.95	0.95	0.95
Mean	0.942	0.957	0.965

TABLE 2

Fringe shifts due to polystyrene and teflon sheets

Sample position (distance in cm. from R2)	Fringe shift (cm.)	
	Polystyrene (0.70 cm.)	Teflon (0.45 cm.)
17.0	0.25	0.05
16.7	0.35	0.12
16.4	0.50	0.30
16.0	0.60	0.35
15.7	0.30	0.12
15.4	0.25	0.05
Mean	0.375	0.165

approximately 2.7. Thus eggs of different quality may not be graded by comparing their dielectric constants. However, their dielectric difference may be so small that the corresponding differences of the fringe shifts falls within the width of a fringe. If this were the case, then, because of no apparent difference of absorption observed in this region of frequency, it might be possible to compare the dielectric constants by measuring the *relative transmitted intensity* in the neighborhood of a fringe. In order to test this possibility, one egg of the lowest albumen quality was first placed on a styrofoam holder between the reflectors and a fringe was located. Then with the reflector and the holder fixed, the transmitted intensities for other eggs were determined. In this experiment, the egg was placed horizontally with its large end toward the source. The results are plotted in Figure 9. Here it must be pointed out that all eggs tested were classified according to their "freshness." It is well known that in general the fresh egg has a smaller air cell and thus larger effective length. In order to eliminate this variation, the same experiment was made with fresh eggs and some treated by a thinning process which utilizes thioglycol [4]. This thinning process can only change the quality of albumen but not the dimension of the air cell. In this case, no apparent grouping of the experimental points, as shown in Figure 9, was obtained. Thus, even though Figure 9 reveals some indication of the egg quality, one cannot conclude that it is definitely due to the dielectric difference.

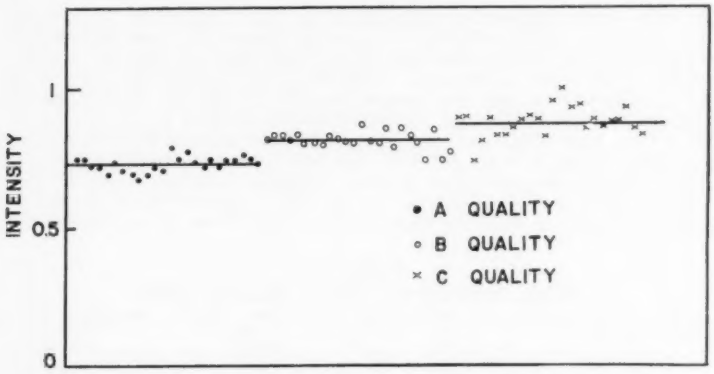

Figure 9. Relative transmitted intensity in the neighborhood of a fringe.

Conclusions. Considering the results stated above, one may conclude that in the microwave region no significant dielectric difference exists between eggs of different albumen quality. A microwave interferometer is therefore useless as an egg albumen classifier.

SUMMARY

Two different microwave techniques were applied to the study of eggs in the search for a reliable correlation between the albumen quality of an egg and some measurable physical property of the egg. The diffraction method is based on the change in the Fresnel zone of diffraction produced by the presence of an egg. This method is very sensitive to the spatial distribution of the dielectric constant inside an egg or to the internal geometry of the egg. The difference in the "geometry" for eggs of different quality appears, however, not to be great enough to allow reliable grading. The microwave interfero-

[4] This process was applied by Dr. L. E. Dawson, Poultry Husbandry Department, Michigan State University, East Lansing.

meter is especially sensitive to changes in the dielectric constant of a substance. The measurements led to the conclusion that in the microwave region a systematic difference in dielectric behavior between eggs of different quality either does not exist or is too small to be measurable.

Acknowledgments

The author wishes to express his sincere gratitude to Dr. E. A. Hiedemann, Dr. R. D. Spence and J. A. Cowen who guided the whole work and gave encouragement constantly. The author is also greatly indebted to Dr. L. E. Dawson for his valuable discussion on the physiological problems, kind cooperation in checking the egg quality and constant interest in the development. During the experiments, hundreds of eggs were used. All of these were supplied by the Poultry Husbandry Department at Michigan State University, to which the author wishes to extend his acknowledgment.

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THERMAL PROCESSING CHARACTERISTICS OF CANNED NON-COMMINUTED MEATS*

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(Manuscript received May 17, 1958)

As stated by Stumbo *(17)* the primary object of thermal processing is to free the food of microorganisms that can endanger the health of a consumer or can cause the food to deteriorate and be unfit for consumption. Jackson and Benjamin *(10)* showed that salting, a well known method of food preservation, can produce, with a thermal process that would be inadequate for the preservation of unsalted product, a commercially sterile product — but from this commercially sterile product viable organisms can be recovered. Salting has long been practiced by meat canners as an element of the processing method used to produce certain commercially sterile cured canned meats. The thermal processes used with these items vary with the packer. Many of these processes leave organisms which are recoverable by conventional bacteriological subculturing. It should be emphasized, however, that in the commercially sterile canned product these organisms do not endanger the health of the consumer nor do they deteriorate the product.

The purpose of this paper is to present data which support the use of Fs concepts *(19)* for developing thermal processes for canned non-comminuted meats. General practice implies destruction of the maximum contamination at the "slowest" heating point in the container and the use of a single point measurement of the heat penetration in multiple samples.

MATERIALS AND METHODS

A. Apparatus and Equipment

Containers used in this experiment were commercial 400 x 512 x 211 pear-shaped cans. They were sealed under 23 to 25 inches of vacuum and were processed in standard commercial retorts at 235°F.

Temperatures were obtained by penetration equipment previously described in detail by Alstrand and Ecklund *(1)* and Ecklund *(7)*. Eight cans were used at each of 2 packing plants for general penetration studies. They were prepared by bottom mounting single Ecklund copper-constantan tube and rod type thermocouples 1¼ in. in length. These were designed to provide within the container only minimum length made from approximately ¼ inch "O.D." rod and the balance, approximately ¾ inch, from approximately ⅛ inch "O.D." rod. They were mounted in countersink type receptacles at a centered point 2-1/16 (201) from the widest end of each can. This type of couple approximates a needle-like contact at the hot junction whereas the previous types of Ecklund couples were blunt or sharply wedged. Both of the latter had a tendency to push and break the meat, causing various imperfections in the seal established between meat and thermocouple. Readings were made at regular intervals using a selector switch and a direct indicating potentiometer.

In addition, special penetration studies were conducted to obtain temperature distribution patterns for this product and the pear-shaped container. These studies were made using varying lengths of Ecklund copper-constantan thermocouples. They were mounted in countersink receptacles with the hot junction as far away from the receptacle as possible. Duplicate determinations were made of 21 different points. Also, a cylindrical

* Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 27, 1958.

can (401 x 211) which had a diameter approximately equal to the width of the pear-shaped can similarly was studied at 9 different points.

B. Product

Cured 1½ lb. commercial ham pieces were obtained at plant "A" (Western Canada) and plant "B" (No. Central U.S.). A total of 176 pieces was packed, with canning and thermal processing being carried out at the plant of origin. After canning, a total of 70 cans—constituting 10 samples from each of 7 separate curing lots—were examined for viable putrefactive anaerobic spores prior to processing. The remaining cans were thermally processed to provide an arbitrary maximum temperature of 210°F., as indicated by the thermocouple readings, at the start of chilling. After chilling, these cans were bacteriologically analyzed.

C. Heat processing

The 1½ lb. ham pieces were check-weighed into 400 x 512 x 211 cans. Pieces most nearly conforming to the can shape were selected for the general heat penetration study. They were closed under vacuum and placed bottom-up in the retort. Thermocouples were connected to insulated copper-constantan leads which in turn were attached to the selector switch. The retort was then closed and an initial temperature reading of each can checked and recorded. Retort operation procedures of the National Canners Association *(11, 12, 16)* were followed to remove air during come-up and prevent accumulation of non-condensable gases during the thermal processing. A process temperature of 235°F. was selected as being in the mid-range of retort temperatures used commercially in the production of commercially sterile canned meats (212-260°F.).

The procedure followed for the special studies was the same as above. Special care was taken to insure a uniform temperature throughout the meat at the start of the thermal process, in order to have comparable initial temperatures at each point being studied.

BACTERIOLOGICAL METHODOLOGY

Hams were sampled by means of sterile triers. Ham plugs were taken at random within the pear-shaped can from the area encompassed by the dimensions of the cylindrical can (401 x 211). Three 10 g. samples were weighed directly into the test medium. Smaller samples were obtained by diluting macerated ham-water emulsions prepared by shaking cut ham plugs in screw capped jars of sterile water containing fragmented glass.

Pasteurization was accomplished by heating the samples to 170°F. and keeping them at that temperature for 15 minutes.

Detection of putrefactive anaerobic spore forming bacteria was accomplished by using liver infusion broth for subculturing in a 3 tube most-probable-number analysis system. Samples and sample aliquots were placed in screw capped 1 x 8 in. tubes containing 50 ml. of the medium.

Liver infusion broth preparation

A. Liver Infusion

1. Grind liver. Add tap water at rate of one liter per pound of liver.
2. Cook 2 hours at 212°F.
3. Strain through cheese cloth to remove particles.
   Particles are placed at 55°C. to dry.

B. Liver Broth

1. To each liter of liver infusion add:

peptone	5.0 g.
tryptone	1.6 g.
dextrose	1.0 g.
soluble starch	1.0 g.
K_2HPO_4	1.25 g.
sodium thioglycollate	0.5 g.

2. Dissolve by heating 30 minutes at 212°F.
3. Adjust pH to 7.4 with 1 N sodium hydroxide.

4. Heat 45 minutes, gradually raising temperature to 250° for the last 10 minutes.
5. Filter through cheesecloth.
6. Dispense in 1 x 8 in. tubes. Add a few dried liver particles to each tube.
7. Sterilize 20 minutes at 250°F.

Tubes were incubated 7 days at 85°F. Presence of putrefactive anaerobic organisms was determined by development of a typical putrid odor in a tube of medium.

Estimation of most probable number of putrefactive anaerobic spore forming bacteria per gram of product was accomplished by referring to the Hoskins *(8)* table.

INTERPRETATION OF HEAT PENETRATION TESTS

General. Data obtained from the general heat penetration tests were treated according to the formula methods developed by Ball *(3, 4)* and the method of Patashnik *(14)*, which is based on the Bigelow, Bohart, Richardson and Ball *(5)* graphical method. These were used to obtain the F_0 of the process at the point of thermocouple measurement. Using the methods developed by Ball, the amount of lethal heat at the thermocouple measuring point was calculated and found to be $F_0 = 0.08$. The Ball method was used, although the cooling curve (fc) did not approximate the (fh) as assumed in the Ball calculation. This anomaly was previously reported by Schultz and Olson *(15)*. Values obtained from the slowest observed heat penetration curve were used in making the calculation below.

BB = 78 minutes. (77 minutes at process temperature plus one minute allowance for 3 minute come-up time.)

$$J = \frac{RT - psIT}{RT - IT} = \frac{235 - (-5)}{235 - 60} = \frac{240}{175} = 1.37$$

$f_h = 110 - 30 = 80$
Come-up time = 3 minutes.

When lethal values computed by Patashnik *(14)* were applied to these data obtained from the heating and cooling curves and totalled, the F_0 value was found to be 0.135. This is illustrated in Table 1.

TABLE 1
Lethal ration values (F/t) for Z = 18° F.

Totalled heating and cooling times		Lethal value per minute	Total lethal values (F_0 values)
At °F	Minutes		
191° - 199°F.	(11)	0.001	0.011
200° - 203°F.	(6)	0.002	0.012
204° - 205°F.	(3)	0.003	0.009
206° - 207°F.	(5)	0.004	0.020
208° - 209°F.	(5)	0.005	0.025
210°F.	(1)	0.006	0.006
211°F.	(4)	0.007	0.028
212°F.	(3)	0.008	0.024
		Grand total =	0.135

Special. Heat penetration studies made to establish distribution patterns were all calculated by the Patashnik *(14)* method. The processing time was kept identical with that used for the general heat penetration study. Also, the F_0 values obtained at many of the points studied varied dependent on the percent and direction of meat shrink during the process. Thus, the slowest heating point was observed to shift along a 1-7/32 inch line drawn through the long axis of the can. Additional studies would be required to define clearly the dimensions for the probable ellipsoidal "cold" area. Figure 1 illustrates the points studied and the observed effect of meat shrink on the F_0 for these points. Points in the narrow end and near the sides of the can had the greatest F_0 variation. Those points

showing variations ranging from approximately 1 or 2 to 5 or 7 were originally in the meat but during the thermal process the meat shrank away leaving the couple in juices outside the meat. The slowest point in the "cold" area shifted but appeared to follow

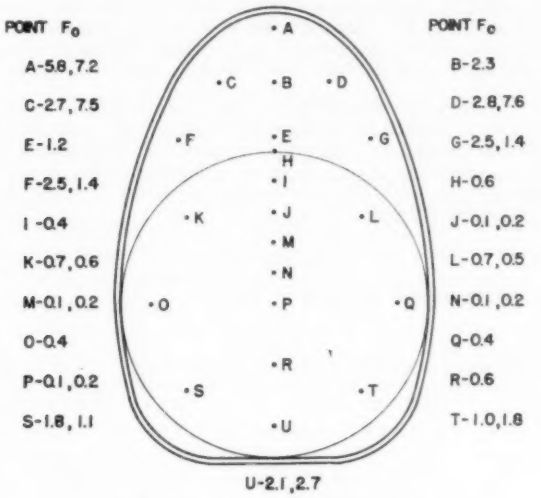

Figure 1. Points studied (top view).

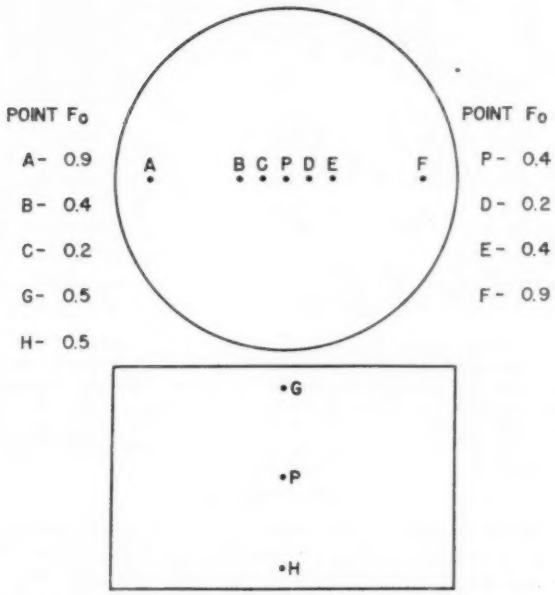

Figure 2. Heat penetration points, top and side views

the center of the chunk of meat; e.g. dependent on the confirmation of the shrinking meat, points J, M, N, or P are equally likely to be the slowest heating point. Although not listed here, actual measurements were made to determine the distance from the respective hot junctions to the meat surfaces. For the points approximately ⅛ in. under the meat surface at the completion of the process, the F_0 ranged from 2 to slightly less than 3, dependent on the general shape and width of the meat at the point of measurement. The circle inscribed within the pear-shaped can is present only as a reference area for the bacteriological data shown in Table 2 and the heat penetration points illustrated in Figure 2.

The pattern studies of the cylindrical can apparently were likewise affected by the extent and direction of shrink. Here too, the processing time was maintained identical with the general heat penetration study. As illustrated in Figure 2 the two slowest heating areas were approximately ¼-inch from the geometric center. From the data, we cannot be certain whether this is due solely to the meat shrink or to the "circular torus" anomaly reported by Hurwicz and Tischer *(9)*.

RESULTS

The bacteriological results are listed in Table 2.

TABLE 2

Viable putrefactive anaerobic spore levels in raw and cooked canned hams

Plant A Lot No.	Raw Product			Cooked Product		
	No. of hams tested	M.P.N. of P.A. spores per gram		No. of hams tested	M.P.N. of P.A. spores per gram	
		Arithmetic Mean	Geometric Mean		Arithmetic Mean	Mean Geometric
1	10	0.0257	0.00921	25	0.00249	0.00092
2	10	0.0224	0.00483	15	0.00070	0.00070
3	10	0.0408	0.02178	10	0.00315	0.00108
4	10	0.0862	0.02290	10	0.00315	0.00108
5	10	0.0106	0.00216	10	0.00070	0.00070
Total	50	0.03714	0.00853	70	0.00206	0.000873
Plant B						
6	10	0.0135	0.00225	10	0.00070	0.00070
7	10	0.0767	0.01047	10	0.00315	0.00100
Total	20	0.0451	0.00486	20	0.00193	0.000837
Grand Total	70	0.0394	0.00732	90	0.00202	0.000854
	Indicated P.A. Spore Destruction During Thermal Processing				94.9%	88.4%

The most probable number values listed in Table 2 are calculated both as arithmetic and geometric means. When averaged arithmetically, the subculture results showed a 94.9% reduction in viable putrefactive anaerobic spores. Thermal destruction of these resistant forms amounted to 94.5% at Plant A and 95.7% at Plant B. The geometric mean values appear to offer the more conservative basis of comparison. On this basis, the P.A. spore kill obtained by the retorting process averaged 88.4%, 89.8% at Plant A and 82.8% at Plant B.

DISCUSSION

The incidence of putrefactive anaerobic spores in the product examined in this study agree with previously published reports by Ayers *et al.*, of very low indigenous P. A. contamination in pork *(6)* and beef *(2)*. Indeed, the raw hams had an average P. A. spore load of less than 4 spores per hundred

grams. Although the plants sampled in this study are separated by approximately 1200 miles, the difference in average putrefactive anaerobic spore level in product packed at the two locations was not significant.

Stumbo, Gross and Vinton *(18)* found that the heat resistance of putrefactive anaerobic spores was the same in cured meat as in uncured meat. For this reason, the authors feel that the data apply to primal canned meats of either character.

From the data presented here, it appears to be obvious that the traditional methods of determining "safe" or "commercially sterile" processing schedules may provide more than adequate lethality in non-comminuted meats. Here, where the product heats almost entirely by conduction, heating the "slowest" point to a fractional F_0 value results in a rigorous cook at the surfaces, which means accompanying high F_0 values. Using the single point method of determining a thermal process at the "slowest" point we would not have predicted the observed spore destruction, nor, it appears, were we certain of measuring the slowest heating point. As described in the section on "Interpretation of Heat Penetration" and as verified in Figure 1, the "cold spot" may be located anywhere within an apparent ellipsoidal area dependent on meat shrinkage during the processing. For accurate measurements, special attention must be paid to placement of the thermocouples. This includes both the actual placement of the couple and also an allowance for the amount and direction of meat shrink due to the thermal process.

It appears that the majority of the indigenous P. A. spore contamination must have been confined to the external surfaces as they alone received sterilization values believed capable of destroying this type of spore *(18)*. Except for these surfaces, the other areas (¼ in. or more beneath the surface) within the prescribed sampling area received thermal destruction values below those generally accepted for P. A. spore destruction (see Figure 1). Thus, although the data do not prove that the indigenous spores were confined primarily to the outer layers, it is contended that this is a logical deduction when the data are viewed in their entirety.

The bacteriological data are explained more adequately by application of the Stumbo Fs theory *(19)* than by the traditional methods *(3, 4)*. These data suggest, moreover, that inoculated pack studies on solid meat pieces should, if the results are to be directly applicable to industrial conditions, confine 90% of the inoculum in the surface layers. Usually, laboratory practice involves the injection of the spore inoculum into the primal piece at several slow heating points, thereby forming a theoretical rather than a normal system.

In this instance, use of the pear-shaped can has made the calculation of unquestionable Fs values a formidable objective. All equations of which we are aware cover calculations of iso-j values *(13)* for cylindrical and/or rectangular containers.

SUMMARY

The Fc (F_0) value of Ball does not appear to describe adequately the spore killing potential of a specific thermal process in canned non-comminuted meats. The Stumbo Fs and layer concepts appear more directly applicable to this type of product. They also may apply in other conduction-heating products where contamination is normally concentrated at the external surfaces.

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THERMAL DESTRUCTION RATES AND REGENERATION OF PEROXIDASE IN GREEN BEANS AND TURNIPS[a, b]

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Peroxidase is one of the most, if not the most, heat resistant enzyme found in plant tissues. Guyer and Holmquist *(7)* summarized data for evaluating the use of high-temperature-short-time sterilization (HTST) principle for canned peas. The times at these temperatures were such that the calculated sterilization values were well above those necessary for sterilization. However, it was noted that off-flavor and odors developed in most samples.

Farkes *et al. (4)* reported that between 212 and 300° F. (100 to 148.8° C.), the heat inactivation curve for peroxidase in green peas indicated that 6 minutes at 250° F. were required to inactivate the enzyme. Esselen and Anderson *(3)* found that in the temperature range of 215° to 290° F. (101.6° to 132.2° C.), the degree of heat required to prevent regeneration of peroxidase activity in vegetable tissues was 2 to 4 times greater than that required to destroy the enzyme on the basis of tests made immediately after heating.

The present investigation is a continuation of studies carried on in this laboratory on the thermal destruction of peroxidase.

EXPERIMENTAL PROCEDURE

A 10-pound portion of fresh green beans was trimmed, cut in one-inch pieces, packed in one-pound polyethylene bags, and frozen overnight. The frozen green beans were then thawed and placed in a Waring blender and comminuted for 5 minutes. The blended green bean puree was strained through 4 layers of cheese cloth. A 15-pound portion of cut and trimmed raw white turnips was frozen overnight and then thawed and sliced. The slices were placed in a Waring blender, mixed with an equal weight of distilled water and blended for 5 minutes. The mixture was strained through 4 layers of cheese cloth.

Method of heating. Samples were heated in a thermoresistometer as described by Pflug and Esselen *(12)* and Esselen and Anderson *(3)*. For operation of the thermoresistometer, at temperatures below 215°F. (101.6°C.), a vacuum pump was connected, Read and Esselen *(14)*, to the side of the steam chamber of the thermoresistometer. No variation in the temperatures of the different pistons was obtained at temperatures as low as 180°F. (82.2°C.).

Quantitative procedure for determination of peroxidase activity. The qualitative test for checking the destruction and survival points of peroxidase in aluminum thermoresistometer cups containing a disc of filter paper *(3)* is a clear test. However, for quantitative analysis, using the method described by Reddi *et al. (15)* which is a modification of Hussein and Cruess *(8)*, it was found that when the cup was rinsed several times with water or buffer (pH 6) and the liquid was transferred to a volumetric flask, the enzyme activity measured was very low. Another method was tried in which the paper disc was transferred to a 10 ml. measuring flask containing 2 ml. buffer of pH 6 but the activity was still low and sometimes absent.

In order to overcome this difficulty, the following method was adopted. A thin layer of glass wool was placed on the bottom of the cup instead of the paper disc. A sample

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[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 27, 1958.

[b] Contribution No. 1163 University of Massachuetts, College of Agriculture, Experiment Station, Amherst, Mass.

of 0.04 ml. of the vegetable juice was placed on the bottom of the cup by using a 0.2-ml. serological pipette graduated in hundredths of a ml. Cups were heated for varying time periods at different temperatures in the thermoresistometer. Contents of 3 cups including the glass wool were transferred quantitatively to a 25 ml. volumetric flask. The flask contained 3 ml. of pH 6.0 citrate phosphate buffer. A 10 ml. aliquot was transferred, by pipette, to a clean test tube and placed in a water bath at 68°F. (20°C.). One ml. of .3% H_2O_2 and 1 ml. of 1% guaiacol (in alcohol) were added. The tube was shaken and placed in a water bath for 50 minutes, and then centrifuged to remove suspended particles of glass wool. The per cent transmission was measured at 450 mμ using a Coleman spectrophotometer, Model 14.

The peroxidase activity was measured immediately after heating to determine the rate of thermal inactivation. The regeneration of peroxidase after heating was determined after storage of the aluminum cups in clean petri dishes at room temperature (70°F.) and at 38°F. Periods of storage were 24 hours at room temperature and 24, 48, 72 and 120 hours at 38°F. A few drops of water were put on the bottom of the petri dishes to prevent desiccation.

Effect of added peroxidase and cytochrome c on off-flavor development in green bean puree. Fifteen pounds of green beans were trimmed, cut in one-inch pieces, and packed in polyethylene bags in one-pound lots. The packed green beans were frozen overnight and then thawed in a water bath at 68° F. (20°C.). The beans were passed through a Fitzpatrick Model M comminuting machine [c] with a screen of 0.036 mesh. The purpose of freezing and thawing before making the puree was to avoid any addition of water to the beans. The puree was mixed, and 20 ml. amounts were packed in 30 ml. dilution bottles with plastic covers. The bottles of green bean puree were sterilized at 250°F. (121.1°C.) for 15 minutes in order to destroy enzyme activity and microorganisms.

The concentration of peroxidase in the fresh green bean puree was determined by the Worthington *(22)* modification of Sumner and Gjessing method *(20)*. It was found that 0.17 peroxidase unit [d] per gram of fresh green beans was present in the puree.

Amounts of horseradish peroxidase [e], which contained 37.5 peroxidase units per milligram as determined by the above procedure, were suspended in sterile distilled water and added to the sterile bean puree in the bottles in a concentration equal to the concentration of peroxidase in fresh green beans. Also, concentrations of 10 times the concentration of peroxidase in fresh green beans were added as above. The experimental packs were prepared as follows:

1. Green bean puree packs with the same concentration of peroxidase and 10 times the concentration as fresh green beans were prepared. The experimental lots of green bean puree were stored at temperatures of 38°F. (3.3°C.), 70°F. (21.1°C.) and 100°F. (37.7°C.) for periods of 1, 2, 4, 6, 8, and 12 weeks. After each time interval samples were removed from storage and examined for off-flavor development, peroxidase activity and total bacteria counts. Controls were sterilized as above and had no peroxidase activity present.

2. A fresh unheated puree of green beans was prepared with added peroxidase to contain 10 times the natural concentration of this enzyme and packed in dilution bottles and stored at 0°F. for periods of 1, 2, 4 and 12 weeks. Samples were examined after storage for peroxidase activity and off-flavor development. In addition a control of fresh green bean puree in dilution bottles was tested as above.

3. Cytochrome c [f] and horseradish peroxidase were added aseptically to dilution bottles containing sterilized green bean puree. The concentration of cytochrome c was 0.005 mg. per g. of green bean puree. Temperatures of storage were 38°F., 70°F., and 100°F. and the periods for organoleptic, peroxidase activity and total bacterial count tests were 0, 1, 2, 4, 6, 8 and 12 weeks. The control was sterilized green bean puree containing cytochrome c.

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[c] Fitzpatrick Model M comminuting machine, The W. J. Fitzpatrick Co., Chicago, Illinois.

[d] Peroxidase unit is that activity which produces one milligram of purpurogallin in 20 seconds.

[e] Prepared and supplied by Worthington Biochemical Corporation, Freehold, New Jersey.

[f] Cytochrome c prepared by Nutritional Biochemical Corporation, Cleveland, Ohio.

Organoleptic tests. A laboratory difference preference test was used in detecting differences and in determining which sample was preferred. Three samples were given to each judge; one was the control and the other 2 samples were the two different concentrations of peroxidase in experiment No. 1. In experiments No. 2 and 3, 3 samples were given to the judges; two were identical representing the control and the third sample was the treatment under investigation. Scores for each sample were given in descriptive terms as: *better than average flavor; average flavor equal to control; below average flavor with no detectable flavor; slightly off-flavor; definitely off-flavor, not acceptable.* The description of off-flavor was requested. A scoring from 1 to 5 was given to these terms, respectively, and data were analyzed using Tukey's method *(21)*. Judges were 10 experienced staff members and graduate students of the Food Technology Department. Before testing green bean puree the bottles were placed in boiling water for 15 minutes and cooled as a safety precaution. Judges were asked to rate flavor and ignore other differences. Suggested words for description of off-flavor were *earthy, flat, fragrant, medicinal, metallic, musty, oily, putrid, puckery, rancid, salty, sour, spicy, sweet,* and *woody.*

Using Tukey's method *(21)* of analysis of variance by ranges, the least significant difference (L.S.D.) was calculated for the 5% level. When a difference between 2 successive average taste panel scores was equal or larger than the L.S.D. value at the 5% level, it could be concluded that the difference established by the panel was a real one due to 95% of variation between samples, and that only 5% was due to a guess on the part of the judges.

Determination of peroxidase activity in experimental packs. After shaking the bottles containing the green bean puree, 2 or 3 ml. of the puree were removed by means of a wide mouth pipette, and filtered through filter paper into a 10 ml. suction flask by using vacuum. A serological pipette graduated in hundredths of a ml. was used to transfer 0.02 ml. of the green bean filtrate to a 10 ml. volumetric flask. One ml. of phosphate citrate buffer (McIlvaine's standard buffer) at pH 6, was added to the flask before making to volume with distilled water. One ml. of phosphate citrate buffer at pH 6, 10 ml. distilled water, and 1 ml. of the above enzyme were added to test tubes. These test tubes were put in a water bath at 68° F. (20° C.) for 5 minutes before starting the reaction. Then 1 ml. of 0.03% hydrogen peroxide was added to each test tube. The zero time of the reaction started from the time of adding 1 ml. of 1% guaiacol in alcohol. Tubes were left for 50 minutes in a water bath at 20° C. Then the per cent transmission was read at a wavelength 450 mμ.

The activity of the peroxidase for each series was considered to be 100 per cent at the beginning of the storage time.

Determination of organic volatile aldehyde in green beans. The accumulation of volatile aldehydes in the tissue of raw and underblanched peas was reported by Joslyn *(9)*. Arighi, Joslyn and Marsh *(1)* found that the total quantity of aldehyde present decreased with a decrease in residual catalase activity.

The finding that high aldehyde content in unblanched or underblanched peas correlated with residual catalase and peroxidase activity as well as with off-flavor formation was reported by Joslyn and Bedford in 1940. After storage for one year at 0°F., peas containing more than 10 p.p.m. of volatile aldehyde, expressed as acetaldehyde were found to be objectionable in flavor (Joslyn and David, *10)*.

Recently Gutterman *et al. (6)* and Lovejoy *(11)* reported that acetaldehyde content closely paralleled off-flavor in frozen asparagus and peas, and they also proposed acetaldehyde content as a measure of off-flavor in frozen asparagus and peas. Acetaldehyde was identified as the chief aldehyde constituent but acetyl methyl carbinol and diacetyl as well as other aldehydes were found.

The procedure of Stotz *(19)* was followed to determine the amount of volatile acetaldehyde present in the packs of green bean puree. The method involves distilling the tungestic acid filtrate of green beans in an all glass unit and collecting the acetaldehyde in bisulfite solution. The p-hydroxybiphenyl color reaction was employed to determine the acetaldehyde. Samples which had no peroxidase were compared with samples that had peroxidase concentration equal to the concentration of peroxidase in fresh green beans, and with samples which had 10 times the concentration of peroxidase in fresh green beans. Recovery of added paraldehyde was 70% under conditions used.

RESULTS AND DISCUSSION

In the thermal destruction tests, use of unheated controls made it possible to express peroxidase content in terms of the per cent remaining after a given heat treatment. A sample composed of the contents of 3 of the 6 cups was used to determine rate of destruction of peroxidase (Tables 1 and 2). The rate of peroxidase destruction regression equations were calculated using

TABLE 1

Thermal destruction rates of green bean peroxidase in the range 180° F. to 260° F. (82.2 to 126.6° C.).

Time of heat treatment in minutes	Per cent of original activity						
	180° F.	190° F.	220° F.	230° F.	240° F.	250° F.	260° F.
0.05	—	—	—	30	22	20	10
0.10	—	—	25	16	11	7	5
0.20	—	—	18	12	11	—	—
0.30	—	—	—	—	—	1	—
0.50	—	—	12	8	0	1	0
1.0	98	96	5	2		0	
1.5	—	—	—	—			
2.0	75	46	3	2			
3.0	—	—	1	0			
5.0	62	51	0				
10.0	11	16					
15.0	15	3					
20.0	9	0					
30.0	11	1					
40	9	0					
56	7						
60	2						
70	0						

TABLE 2

Thermal destruction rates of turnip peroxidase in the range 220 to 285° F. (104.4 to 140.5° C.).

Time of heat treatment in minutes	Per cent of original activity					
	220° F.	230° F.	240° F.	250° F.	270° F.	285° F.
0.05	—	40	44	20	14	0
0.10	56	46	20	12	10	0
0.20	—	—	—	10	3	
0.30	—	42	—	—	—	
0.50	—	42	16	5	1	
1.0	48	38	10	2	0	
1.50	—	38	—	—		
2.0	40	29	8	0		
3.0	—	25	8			
5.0	30	20	5			
7.0	26	—	0			
10.0	11	5				
15.0	10	—				
20	10	1				
30	7	0				
40	2					
50	1					
60	0					

the procedure outlined by Bliss *(2)*. The D values[6] (Table 3) for green beans and turnips were obtained by finding the reciprocal of the slope, or b, factor from the regression equations. Thermal destruction time (TDT) curves were derived by plotting the log of D against the corresponding temperature (Figures 1 and 2).

[6] D = the temperature to which the actual D value applies is usually indicated by a subscript — e.g. D_{220} or D_{250} equals for 90% destruction at 250° F.

TABLE 3

Times for 90 per cent destruction (D) for peroxidase in turnips and green beans heated at temperatures in the range 180° F. to 270° F. (82.2 to 132.2° C.).

	Temperatures ° F.						
	180 D Min.	190 D Min.	220 D Min.	230 D Min.	240 D Min.	250 D Min.	270 D Min.
Turnips			40.0	12.3	6.8	1.8	0.35
Green beans	20.9	10.3	2.9	1.5	0.8	0.40	

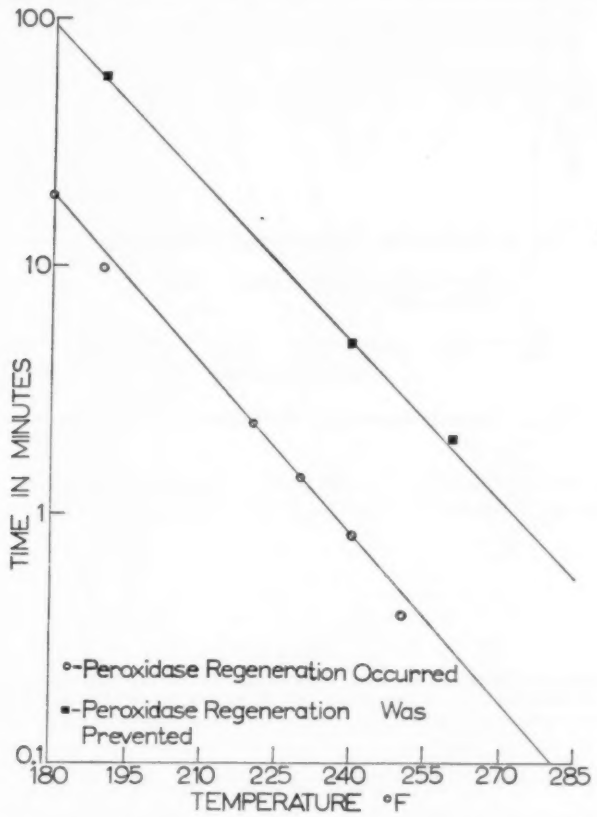

Fig. 1. Thermal destruction time curves of green bean peroxidase.

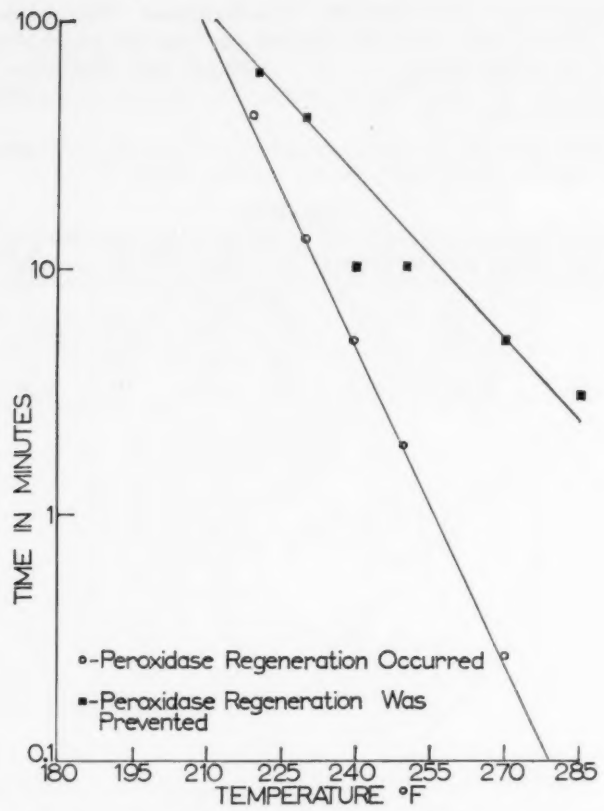

Fig. 2. Thermal destruction time curves of turnip peroxidase.

The velocity coefficient, k, expressed in reciprocal time units (Table 4) was determined graphically from the D value through use of the equation

$$k = \frac{2.3}{D}$$

TABLE 4

Velocity coefficient (k) for peroxidase destruction in turnips and green beans at temperatures in the range 180° F. to 270° F. (82.2 to 132.2° C.).

	Temperatures ° F.						
	180	190	220	230	240	250	270
	K min.$^{-1}$	*K min.*$^{-1}$	*K min.*$^{-1}$	*K min.*$^{-1}$	*K min.*$^{-1}$	*K min.*$^{-1}$	*K min.*$^{-1}$
Turnips			0.0575	0.1869	0.3382	1.2777	6.5710
Green beans	0.1100	0.2233	0.7931	1.5333	2.8750	5.7500	——

This equation was derived from the differential equation: $-(dc/C)dt = k$, where $-(dc/C)$ represents the fractional decrease in concentration C, occurring during the time dt. The integrated form of the equation is based on the 90 per cent level of destruction, as indicated by the use of the D value.

The relationship between the peroxidase destruction and temperature was obtained from a plot of the reciprocal of the absolute temperature. A resultant straight line is taken to indicate that the reaction satisfies the Arrhenius equation:

$$\text{Log}\ \frac{k_2}{k_1} = \frac{E\ (T_2 - T_1)}{2.3\ R\ (T_1\ T_2)}$$

where E is the activation energy, k_2 and k_1 are rates of T_2 and T_1 (Kelvin), R is the gas constant and equals 1.98, and 2.3 is the natural logarithm.

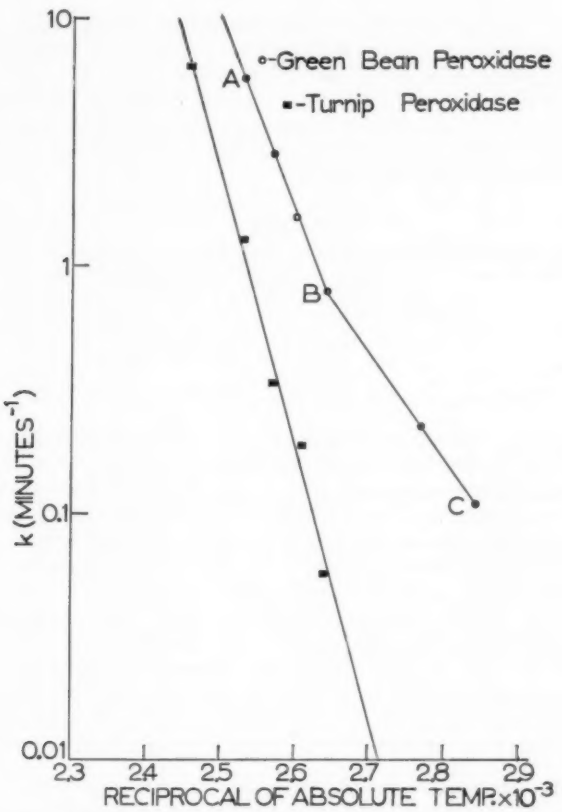

Fig. 3. Plot of the velocity coefficient against the reciprocal of the absolute temperature for turnip and green bean peroxidases.

The value of E obtained from Figure 3 was found to be 34.4 K calorie and 19.6 calorie for the parts of the curve AB and BC in green beans, respectively. The velocity of the reaction increased with the increase in temperature. The critical temperature was 220° F. (104.4° C.) for this change of velocity. For the turnips the value of E was found to be 49 K calorie.

Q_{10} was calculated from the relationship

$$Q_{10} = \frac{Kt}{K(t\text{-}18)}$$

in which K(t) is the reaction rate constant at temperature T_2 and K(t-18) is the reaction rate constant at a temperature 18° F. (10° C.) below T_2.

For the peroxidase of green beans Q_{10} values of 2 and 7.8 were found, respectively, for the 2 velocities of reaction. The Q_{10} for the peroxidase of turnips was 20.9. From these results it is clear that peroxidase destruction under 220° F. was doubled every 18° F. (10° C.).

Figures 1 and 2 show the thermal destruction time (TDT) curves for peroxidase of both green beans and turnips. The slopes of these curves, or z[h] values, were found to be 41° F. and 23° F., respectively. It is observed from Table 5 and Figures 1 and 2 and F_{212}[i] and F_{250}[j] values for thermal destruction time curves of peroxidase that prevent regeneration in green beans and turnips are 5 to 6 times higher than these values required to destroy the enzyme when tested immediately after heating.

TABLE 5

Thermal resistance characteristics of peroxidase in green beans and turnips.

	Inactivation after heating			Inactivation to prevent regeneration		
	212° F.	250° F.	z	212° F.	250° F.	z
	Min.	*Min.*	° *F.*	*Min.*	*Min.*	° *F.*
Green beans	3.7	0.48	41	20	3.0	47
Turnips	73	1.8	23	95	11.3	46

Regeneration rates of peroxidase were not constant during storage (Tables 6, 7, 8 and 9). Rossoff and Cruess *(16)* in their work on the oxidase of cauliflower reported that the inconsistency of regeneration in their work may have been due to irregularity in re-solubility of the heat-coagulated proteins with which the enzyme was associated. The reason for the high initial regeneration rate followed by the decreased rate is not known, but a possible explanation based on the theory of Fischer *(5)* is: Upon heating, the enzyme is partially denatured, the protein fraction being precipitated. This precipitated protein absorbs and protects from heat some of the remaining undenatured enzyme. During the first few days of storage, the undenatured enzyme is eluted from the denatured protein and again becomes active in solution. Then the denatured protein, according to the postulation of Schwimmer *(17)*, regenerates at a slow rate to give an increase in peroxidase activity. The longer the heat

[h] The slope of thermal destruction time curve expressed as the number of degrees F required for the curve to pass through one log cycle on semilogarithmic paper.

[i] F_{212} = the number of minutes to destroy the peroxidase activity at 212°F.

[j] F_{260} = the number of minutes required to destroy the peroxidase activity at 250°F.

TABLE 6

Thermal inactivation of peroxidase in green beans, determined immediately after heating and its regeneration after 24 hours at room temperature.

Time of heat treatment in minutes	Temperature of treatment															
	180° F.		190° F.		220° F.		230° F.		240° F.		250° F.		260° F.		270° F.	
	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.
0.05	—	—	—	—	—	—	30	42	22	16	20	13	10	4	0	0
0.10	98	100	96	42	25	30	16	27	11	9	7	9	5	2		
0.20	—	—	—	—	18	23	12	21	11	3	—	—	—	—		
0.30	—	—	—	—	—	—	—	—	—	—	1	9	—	—		
0.50	—	—	—	—	12	18	8	17	0	3	1	4	0	2		
1.00	—	—	—	—	5	8	2	4	0	3	0	6	0	0		
1.50	—	—	—	—	—	—	—	—	—	—	0	3				
2.00	75	82	46	31	3	8	2	4	0	2	0	0				
2.50	—	—	—	—	—	—	0	2	0	2						
3.00	—	—	—	—	1	2	0	0	—	—						
4.00	—	—	—	—	1	2			—	—						
5.00	62	51	51	16	—	—			0	0						
6.00	—	—	—	—	0	2										
8.00	—	—	—	—	0	2										
10.00	11	25	16	16	0	0										
15.00	15	25	3	7												
20.00	9	11	1	3												
30.00	11	4	0	3												
40.00	9	2	0	3												
50.00	7	4	0	3												
60.00	2	2	0	3												
70.00	0	0														

TABLE 7

Thermal inactivation of peroxidase in turnips determined immediately after heating and its regeneration after 24 hours at room temperature.

Time of heat treatment in minutes	Temperature of treatment											
	220° F.		230° F.		240° F.		250° F.		270° F.		285° F.	
	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.	0	24 hrs.
0.05	—	—	40	72	44	60	20	50	0	22	0	0
0.10	50	66	46	58	20	52	12	44	0	0		
0.20	—	—	—	—	22	30	10	11				
.30	—	—	42	58	—	—	—	—				
.50	—	—	42	60	16	20	5	11				
1.0	43	53	38	56	10	16	2	0				
1.5	—	—	38	46	—	—	—	—				
2.0	40	60	29	56	8	15	0	0				
3	—	—	25	50	8	10						
5	28	46	20	40	5	8						
7	22	38	—	—	0	8						
10	10	31	5	17	0	0						
15	7	22	—	—								
20	—	17	1	6								
25	—	—	0	4								
30	5	6	0	4								
35	0	2	—	—								
40	0	0	0	0								

TABLE 8

Typical data of thermal destruction and regeneration of green bean peroxidase at 220° F. (104.4° C.).

Time of heat treatment in minutes	Per cent of original activity				
	Storage time in hours at 38° F.				
	0	24	48	72	120
0.10	25	32	30	26	29
0.20	18	22	22	22	24
0.50	12	16	14	16	16
1.00	5	6	6	6	10
2.00	3	0	2	2	6
3.00	1	1	2	2	2
4.00	1	2	2	2	2
6.00	0	2	2	2	2
8.00	0	0	0	0	0

treatment the greater the denaturation and the less possible is the regeneration of undenatured enzyme during the first few days of storage. The observed results in this investigation are in agreement with this theory. The repair of the enzyme molecules needs more time in samples heated for longer times at higher temperatures than samples heated for shorter times at lower temperatures.

There is no satisfactory explanation for the sharp increase of activation energy of peroxidase of green beans at a critical temperature (220° F.) but it might be suggested, as Sizer *(18)* did, that this transition corresponds to a shift in the enzyme molecule from one configuration to another.

By comparing the pH values of green bean puree in Table 10 with each other it can be observed for all samples that their pH values decreased during

TABLE 9

Typical data of thermal destruction and regeneration of turnip peroxidase at 230° F. (110° C.).

Time of heat treatment in minutes	Per cent of original activity				
	Storage time in hours at 38° F.				
	0	24	48	72	120
0.05	40	75	73	61	63
0.10	46	65	58	60	75
0.30	42	58	48	51	58
0.50	42	57	55	30	52
1.00	38	50	37	42	48
1.50	38	48	47	42	—
2.00	29	28	33	43	37
3.00	25	30	23	32	37
5.00	20	25	20	18	25
10.00	5	18	16	13	11
20.00	1	3	2	4	4
25.00	0	0	0	4	4
30.00	0	0	0	4	4
40.00	0	0	0	0	0

TABLE 10

pH values for green bean puree during storage.

Experiment	0 time fresh	0 time after processing	8th week	12th week
I A_1	6.00	5.70	4.53	4.50
I B_1	6.00	5.70	4.30	4.45
I A_1 Control	6.00	5.70	5.50	5.45
I A_2	6.00	5.70	4.27	4.35
I B_2	6.00	5.70	4.08	4.25
I A_2 Control	6.00	5.70	5.40	5.20
I A_3	6.00	5.70	4.65	4.70
I B_3	6.00	5.70	4.42	4.50
I A_3 Control	6.00	5.70	5.33	5.25
II A	6.00		—	5.30
II A Control	6.00		—	5.63
III A_1	6.00	5.70	4.45	4.45
III B_1	6.00	5.70	5.55	5.25
III A_2	6.00	5.70	4.25	4.30
III B_2	6.00	5.70	5.45	5.20
III A_3	6.00	5.70	4.83	4.75
III B_3	6.00	5.70	5.30	5.10

I A_1, I A_2 and I A_3 = Sterilized green bean puree samples containing the same concentration of peroxidase as in fresh green beans stored at 38, 70 and 100° F., respectively.

I B_1, I B_2 and I B_3 = Sterilized green bean puree samples containing 10 times the concentration of peroxidase as in fresh green beans stored at 38, 70 and 100° F., respectively.

II A and II B = Fresh green bean puree samples containing 10 times the concentration of peroxidase as in fresh green beans stored at 38 and 0° F., respectively.

III A_1, III A_2 and III A_3 = Sterilized green bean puree samples containing the same concentration of peroxidase as in fresh green beans stored at 38, 70 and 100° F., respectively.

III B_1, III B_2 and III B_3 = Sterilized green bean puree samples plus cytochrome c stored at 38, 70 and 100° F., respectively.

sterilization. For all samples that had peroxidase in the same concentration as the natural green beans the decrease was less than the samples that had 10 times the concentration of peroxidase. The decrease was more pronounced in samples stored at 70° F. than at 38° F. and 100° F.

Table 11 shows that perioxidase added in the same concentration as is naturally present in green beans was destroyed at 6 weeks when stored at 38° F., 8 weeks when stored at 70° F. and 50% residual peroxidase was present at 12 weeks when stored at 100° F. For samples that had 10 times the natural concentration of peroxidase, the activity of peroxidase decreased in samples stored at 70° F. and 100° F. more than in samples stored at 38° F. The organoleptic tests indicated (Table 12) that off-flavor in the green bean puree was due to peroxidase activity.

In a recent paper by Purr *(13)* the phenomenon of regeneration of peroxidase was considered as being an effect of the cytochrome c content of the plant material. If the cytochrome c was destroyed, no regeneration was noticed. If there was a cytochrome c present after heating, a substantial degree of regeneration was observed. In this investigation it was observed that the presence of cytochrome c in green bean puree, containing the natural concentration of peroxidase, caused inhibition or destruction of the peroxidase activity. The off-flavor was pronounced in samples that had added peroxidase and cytochrome c. Samples which had added cytochrome c only were acceptable by

TABLE 11

Peroxidase activity during storage of green bean puree.

Sample	Per cent peroxidase activity						
	0 Time	1st week	2nd week	4th week	6th week	8th week	12th week
I A_1	100	109	60	33	00	00	00
I B_1	100	110	108	76	92	80	80
I A_2	100	80	20	20	20	00	00
I B_2	100	100	92	80	65	60	37
I A_3	100	81	72	60	45	50	54
I B_3	100	95	80	80	53	40	32
II A	100	107	110	190	—	—	180
II B	100	26	16	Moldy	—	—	—
III A_1	100	00	00	00	00	00	00
III B_1	100	00	00	00	00	00	00
III A_2	100	22	13	18	00	00	00
III B_2	100	00	00	00	00	00	00
III A_3	100	00	00	00	00	00	00
III B_3	100	00	00	00	00	00	00

I A_1, I A_2 and I A_3 = Sterilized green bean puree samples containing the same concentration of peroxidase as in fresh green beans stored at 38, 70 and 100° F., respectively.

I B_1, I B_2 and I B_3 = Sterilized green bean puree samples containing 10 times the concentration of peroxidase as in fresh green beans stored at 38, 70 and 100° F., respectively.

II A and II B = Fresh green bean puree samples containing 10 times the concentration of peroxidase as in fresh green beans stored at 38 and 0° F., respectively.

III A_1, III A_2 and III A_3 = Sterilized green bean puree samples containing the same concentration of peroxidase as in fresh green beans stored at 38, 70 and 100° F., respectively.

III B_1, III B_2 and III B_3 = Sterilized green bean puree samples plus cytochrome c stored at 38, 70 and 100° F., respectively.

TABLE 12

Green bean puree with added peroxidase stored at 38, 70 and 100° F.

Sample	Storage time in weeks	Storage temperature 38° F.		Storage temperature 70° F.		Storage temperature 100° F.	
		Taste score	Flavor and odor	Taste score	Flavor and odor	Taste score	Flavor and odor
I A	0	N.S.D.	N.S.D.** Acid cooked flavor	N.S.D.	N.S.D. Acid cooked flavor	N.S.D.	N.S.D. Acid cooked flavor
I B	0		Acid cooked flavor		Acid cooked flavor		Acid cooked flavor
Control	0		Acid cooked flavor		Acid cooked flavor		Acid cooked flavor
I A	1		S.D. Acid cooked flavor		N.S.D. Strong, acid		S.D. Acid cooked flavor
I B	1		*Hay-like, stale flavor		Hay-like		Slight acid, hay-like
Control	1	S.D.	Acid cooked flavor	S.D.	Acid cooked flavor	S.D.	Acid cooked flavor
I A	2		S.D. Acid cooked flavor		S.D. Stale,		S.D. Stale,
I B	2		*Stale,		N.S.D. Strong, acid		N.S.D. Acid
Control	2	S.D.	Acid cooked flavor	S.D.	Acid cooked flavor	S.D.	Acid cooked flavor
I A	4				S.D. Strong, stale & acid		S.D. Slight acid
I B	4	S.D.	N.S.D. Sour	S.D.	N.S.D. Strong, stale & acid	S.D.	N.S.D. Strong stale, bitter
Control	4		Stale, acid		Acid cooked flavor		Acid cooked flavor
I A	6				S.D. Strong acid		S.D. Stale, oxidized
I B	6	S.D.	N.S.D. Stale, oxidized	S.D.	N.S.D. Very strong acid	S.D.	N.S.D. Slightly off
Control	6		Stale, too acid		Acid cooked flavor		Acid cooked flavor
			Acid cooked flavor				
I A	8				S.D. Sour, strong, stale		S.D. Strong stale, slight acid
I B	8	S.D.	N.S.D. Sour, stale	S.D.	N.S.D. Strong acid	S.D.	N.S.D. Sour, off-flavor
Control	8		Sour, stale		Stored acid cooked flavor		Stored acid cooked flavor
			Acid cooked flavor				
I A	12				S.D. Sour, strong stale		S.D. Strong stale, slight acid
I B	12	S.D.	N.S.D. Sour, stale	S.D.	N.S.D. Strong acid, not stale	S.D.	N.S.D. Sour, off-flavor
Control	12		Sour, stale		Stored acid cooked flavor		Stored acid cooked flavor
			Stored acid cooked flavor				

* S.D. = Significant difference at 5% level.
** N.S.D. = No significant difference at 5% level.
1 A = Sterilized green bean puree containing the same concentration of peroxidase as in fresh green beans.
1B = Sterilized green bean puree containing 10 times the concentration of peroxidase as in fresh green beans.

the taste panel. This gives further evidence that although peroxidase activity was inhibited or diminished by either increased acidity or the presence of an inhibitor or both, off-flavor appeared due to biochemical reactions which were started by the addition of peroxidases.

Presence of peroxidase caused an increase in the acetaldehyde content of the samples (Table 13).

TABLE 13

Organic volatile acetaldehyde in green bean puree stored at room temperature.

Sample	Storage Time in Weeks			
	0 time acetaldehyde concentration	2 weeks acetaldehyde concentration	4 weeks acetaldehyde concentration	12 weeks acetaldehyde concentration
	mg. %	*mg. %*	*mg. %*	*mg. %*
Fresh green bean puree	1.4			
Puree of sterilized green bean (control)	0.84	1.0	1.4	1.2
Puree of sterilized green beans and has equal concentration of H.R.P. as in fresh green beans		3.4	2.4	2.6
Puree of sterilized green bean puree has 10 times concentration of H.R.P. as in fresh green beans		8.0	5.3	5.5

SUMMARY

A quantitative method for determining the peroxidase activity after heating 0.04 ml. samples in the cup of the thermoresistometer was developed.

Thermal inactivation curves for peroxidase of green beans and turnips possessed z values of 41.0 and 23.0° F and F_{250} values of 0.48 and 1.8 respectively. The thermal destruction curves required to prevent regeneration of peroxidase for green beans and turnips show z values of 47 to 46° F. and F_{250} values of 3.0 and 11.3, respectively. These z and F_{250} values are 5 to 6 times higher than those required to inactivate the enzyme when measured immediately after heating.

The activation energies for green bean peroxidase were found to be 34.4 and 19.6 K calorie with a critical temperature at 220° F.; the activation energy for turnip peroxidase was found to be 49 K calorie.

Regeneration of green bean and turnip peroxidases occurred when heated samples were stored at room temperature for 24 hours. Regeneration did not occur at a constant rate when samples were stored at 38° F. for periods up to 120 hours.

Experimental packs of sterilized green bean puree containing the natural concentration and 10 times this amount of peroxidase showed off-flavors and odors when compared with sterilized control samples of green bean puree kept at 38, 70 and 100° F. for 12 weeks.

Peroxidase activity decreased in the experimental samples during storage. The decrease was more rapid for samples stored at 70 and 100° F. than for those stored at 38° F. A decrease of almost one pH unit was observed in samples with added peroxidase. An increase of acetaldehyde due to the

presence of peroxidase activity was noted. The acetaldehyde content varied from 2 to 5 times that present in fresh green beans.

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COMPARATIVE STUDIES ON MEDIA FOR COUNTING ANAEROBIC BACTERIAL SPORES[a]

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Investigations described in this paper are related entirely to the recovery of viable spores of PA-3679 and *Clostridium botulinum* type B. Spores of the respective test organisms were subjected to thermal treatment and gamma radiation independently. Questionnaires were distributed in January, 1957, to 26 individuals at various laboratories whose personnel have been interested in spore recovery after exposure to either heat and/or radiation. Information relative to recovery media used in counting anaerobes was requested. The cooperation of the recipients[b] was appreciated as it permitted the study of a rather broad spectrum of media.

A widespread interest in counting anaerobic spores, particularly thermally treated spores, has been manifested since the early 1930's. At processing conferences held in conjunction with the annual convention of the National Canners Association, the subject has been regularly discussed. Periodic attempts have been made to procure uniform procedures for thermal death time studies, and spore counting has been one of the several facets.

In a restricted National Canners Association Report *(2)*, data were presented based on the cooperative work of 7 laboratories. The type of media employed was only one of several variables investigated. The Institute of Food Technologists has for some years been interested in this phase along with other problems related to thermal death time work. In 1952 the Western Utilization Research Branch, USDA, conducted a survey on spore counting media with *Clostridium botulinum* and the results were distributed in private communications.

Frank and Campbell *(1)* reported that the recovery media employed in a study with PA-3679 had an effect on the thermal resistance values. Six media with modifications were studied.

Replies to our questionnaire listed 20 different media. It was indicated that they are used under varying conditions, such as spore counts on processed and non-processed foods, as well as thermal death time work. Several were used for Most Probable Number determinations, others for colony counting, and others merely for the development of growth as indicated by gas formation with odor.

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[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 27, 1958.

[b] Information on the subject was sought from the following universities: California, Chicago, Illinois, Iowa State, Massachusetts, Michigan, Texas (at Austin and Houston), and the State College of Washington. The Government agencies to whom inquiries were sent were the U.S.D.A., Eastern and Western Utilization Branches, the Bureau of Human Nutrition, and the QM Food and Container Institute for the Armed Forces. The following were contacted in industry and related agencies: Armour & Co., Continental Can Co., Food Machinery & Chemical Co., General Mills, H. J. Heinz Co., Hormel Institute, Mead-Johnson & Co., John Morrell & Co., National Canners Assoc. (Eastern and Western Laboratories), Swift & Co., and the American Can Company's Technical Service Division.

TABLE 1
Media Variations

Medium No.	Agar media	$NaHCo_3$	Sterilization	Tube stratification	Incubation temperature
1	Peptone colloid	None	15 min./250° F.	None	98° F.
2	Eugonagar	.5 ml. — 10%	15 min./243° F.	None	"
3	Beef infusion	None	20 min./250° F.	Yes	86° F.
4	Tryptone yeast extract	.2 ml. — 10%	15 min./250° F.	Yes	"
5	Pork infusion	None	20 min./250° F.	Yes	"
6	Pork-pea infusion	.4 ml. — 5%	12 min./250° F.	Yes	"
7	Thioglycollate w/o dextrose	None	15 min./250° F.	None	98° F.
8	Brain-heart infusion with PAB	None	15 min./250° F.	None	"
9	T BEST	None	15 min./250° F.	None	86° F.
10	Beef extract	.2 ml. — 10%	15 min./250° F.	Yes	98° F.
11	Beef liver infusion	.5 ml. — 5%	15 min./250° F.	Yes	86° F.

A wide diversification was noted in media sterilization, methods of preparation, methods of clarification, incubation times and temperatures, methods of culturing, etc. Not infrequently more than one medium was used by a given laboratory. A summary of 11 media used in this investigation is given in Table I, depicting some of the variations reported.

The scope of this investigation may be divided as follows:

Phase I.

Recovery of heated spores of PA-3679 in 10 media after both one and two sterilizations of these media, with incubation at 86° F.

1. Severely heated spores (Tables 2 and 8).
2. Mildly heated spores (Tables 3 and 8).

TABLE 2
Recovery Counts PA-3679 — Severely Heated Spores

Medium	Total counts from 5 — 1 ml. aliquots					
	Experiment 1 [1]		Experiment 2 [2]		Experiment 3 [3]	
	Sterilized once	Sterilized twice	Sterilized once	Sterilized twice	Sterilized once	Sterilized twice
1	0	0	0	0	Not included.	
2	0	0	0	0	"	"
3	205	252	84	76	130	75
4	35	62	26	27	37	11
5	136	196	64	73	98	68
6	245	261	88	95	135	100
7	0	1	0	0	Not included.	
8	87	63	20	30	50	21
9	0	0	0	0	Not included.	
10	79	111	40	39	44	18

[1] Spores processed 2′ 30″ @ 250° F. for media sterilized once. Spores processed 2′ 15″ @ 250° + F. for media sterilized twice.
[2] Spores processed 2′ 30″ @ 251° F. throughout experiment 2.
[3] Spores processed 2′ 30″ @ 251 + °F. throughout experiment 3.

TABLE 3
Recovery Counts PA-3679 — Mildly Heated Spores

Medium	Total counts from 5 — 1 ml. aliquots			
	Experiment 5 [1]		Experiment 6 [1]	
	Sterilized once	Sterilized twice	Sterilized once	Sterilized twice
1	3T	T [2]	T	T
2	34	59	81	59
3	33	52	48	52
4	72	82	72	82
5	60	37	60	37
6	116	90	76	90
7	32T	T	T	T
8	19	17	21	1T
9	32T	23	34	23
10	68	76	68	76

[1] Spores processed 27″ @ 220° F.
[2] T = turbidity prevented complete counting.

Phase II.

1. Recovery of severely heated spores of PA-3679 in 3 infusion media, each prepared from three separate batches of infusion from different lots of raw product (Tables 4 and 9), with incubation at 86° F.

2. Phase II, part 1, was repeated using 2 strains of PA-3679 with a slight difference in thermal resistance and grown in Difco egg-meat medium (Tables 5 and 9) furnished by Dr. C. F. Schmidt.

TABLE 4

Recovery Counts PA-3679 — Infusion Variations

Medium	Total counts from 5 — 1 ml. aliquots								
	Infusions								
	Batch 1			Batch 2			Batch 3		
Runs	# 7 [1]	# 8 [1]	# 9 [2]	# 7 [1]	# 8 [1]	# 9 [2]	# 7 [1]	# 8 [1]	# 9 [2]
3.....	170	85	137	155	71	145	129	54	96
5.....	124	59	94	143	52	100	131	65	85
6.....	143	77	123	170	58	133	159	76	99

[1] Spores processed 2′ 30″ @ 251° F.
[2] Spores processed 3′ 18″ @ 251° F.

TABLE 5

Recovery Counts PA-3679-42-43 — Infusion Variables

Susp. No.	Medium	Total counts from 5 — 1 ml. aliquots					
		Infusions					
		Batch 1		Batch 2		Batch 3	
	Runs	# 10 [1]	# 11 [1]	# 10 [1]	# 11 [1]	# 10 [1]	# 11 [1]
3679-42	3	72	38	59	47	42	35
"	5	44	33	41	50	37	40
"	6	89	83	89	89	87	68
		# 12 [2]	# 13 [3]	# 12 [2]	# 13 [3]	# 12 [2]	# 13 [3]
3679-43	3	28	8	33	14	17	4
"	5	24	2	17	7	26	5
"	6	39	14	42	16	46	15

[1] Spores processed 2′ 42″ @ 250° F.
[2] Spores processed 2′ 30″ @ 250° F.
[3] Spores processed 2′ 30″ @ 251+° F.

Phase III.

Recovery of irradiated spores in eleven media incubated at 86° F. and at 98° F.

1. PA-3679 receiving high and low radiation doses (Tables 6 and 10.)

2. *Cl. botulinum,* type B-one "radioresistant" strain and one "radiosensitive" strain (Tables 7 and 10).

TABLE 6
Recovery Counts of Irradiated Spores of PA-3679

Medium	Total counts from 5 — 1 ml. aliquots				
	540,000 rads average				1,540,000 rads
	Experiment #14		Experiment #16		Experiment #15
	86° F.	98° F.	86° F.	98° F.	86° F.
1	T [1]	T	4	10	2
2	141	192	28	29	15
3	47	70	14	41	8
4	73	122	19	26	4
5	62	78	24	41	13
6	81	118	33	33	11
7	T	T	10	11	11
8	26	32	14	32	14
9	T	T	22	31	10
10	57	57	29	32	15
11	61	88	39	48	8

[1] T = turbid. Reformulation with additional agar permitted counts on subsequent experiments.

TABLE 7
Recovery Counts of Type B Clostridium botulinum Spores Irradiated at 500,000 rad

Medium	Total counts from 5 — 1 ml. aliquots			
	Strain 13983B		Strain 32B	
	86° F.	98° F.	86° F.	98° F.
1	6	8	4	10
2	42	32	28	29
3	32	38	14	41
4	34	49	19	26
5	22	45	24	41
6	64	88	33	33
7	7	9	10	11
8	42	33	14	32
9	18	21	22	31
10	46	53	10	32
11	21	28	39	48

TABLE 8
Comparison of Media Based on Total Log Counts PA-3679

Medium	Severe heat			Mild heat	
	Experiments			Experiments	
	1 — 1A	2 — 2A	3 — 3A	5 — 5A	6 — 6A
2				9.9	11.3
3	16	12	12	9	10
4	9	6.5	6	11	11.8
5	15	11	11	10.3	9.6
6	17	12.5	13	13	12
8	11	6.7	7	5	5
10	12	8	7	10.2	11.4
	L.S.D. = 1.6			L.S.D. = 1.4	

Note: L.S.D. is the least significant difference at the .05 significance level(3).

TABLE 9
Comparison of Media Infusion Based on Total Log Counts PA-3679

Medium	PA-3679 Experiments			PA-3679-42 Experiments		PA-3679-43 Experiments	
	7	8	9	10	11	12	13
3	22	16.3	20.3	15.6	13.4	10	3
5	21.1	15.1	18.8	13.4	13.2	9	0
6	22.3	17	20.8	18.6	17.8	13.5	6
				L.S.D.= 8		L.S.D.= 8	

Note: L.S.D. is the least significant difference at the .05 significance level(3).

TABLE 10
Comparison of Media Based on Total Log Counts for Irradiated Spores

Susp.	PA-3679	PA-3679	PA-3679	Bot. 13983B	Bot. 32B
Dose	.54 Mrad	1.54 Mrad	.54 Mrad	.5 Mrad	.5 Mrad
Experiment	14	15	16	17	18
Medium					
1		0.9	0.5	1.2	1.1
2	15	1.7	6.2	8.6	7.2
3	10.2	0.8	0	8.1	6.2
4	12.6	− 0.3	4.2	9.0	6.0
5	11.3	1.7	1.3	7.1	7.6
6	12.9	1.5	1.9	11.7	7.9
7		1.6	0.9	1.7	2.9
8	7.5	2.2	1.3	8.5	6.1
9		1.3	2.0	5.4	5.7
10	10.5	2.3	1.0	9.9	7.5
11	11.5	− 0.7	3.5	6.8	9.2
L.S.D.	1.73	0.9	1.73	1.92	1.92

Note: L.S.D. is the least significant difference at the .05 significance level(3).

METHODS AND PROCEDURES

Organisms. Spore suspensions of PA-3679 (except 3679-42 and 43) and *Cl. botulinum* type B were grown in a beef heart infusion formulation at 98°F. Spores were centrifuged and the supernatant liquor decanted, leaving a very small portion in which to suspend the spores. The spores were heated 10 minutes at 170°F., cooled, and then held at 36°-40°F.

Thermal processing. For all phases involving heat, a sufficient number of spores were suspended in phosphate buffer (pH 7.0) to yield a readily countable number after processing, which was carried out with the thermoresistometer.

One-hundredth ml. portions of the prepared suspensions were placed in standard cups and aluminum boats, which in turn were placed in 5 of the carrying slots or chambers (Figure 1). A sixth cup containing 0.01 ml. of sterile water was placed in the 6th slot to serve as a control. All cups, after heating, fell individually into tubes (Figure 2) holding 12 ml. of sterile water. Culturing for viable spores was done from these tubes (Figure 3) after the samples were thoroughly shaken. Severe heat treatment was exposure in the sterilizing chamber for 2 minutes, 30 seconds at 250°-251°., whereas the milder exposure was 27 seconds at 220°F.

In experiments conducted under Phase II, the procedure was changed, in that the contents of all 5 collection tubes were pooled and the subculturing done from this composite sample. This change of technique was employed in order to eliminate possible irregular heating errors in the individual slots or chambers of the thermoresistometer.

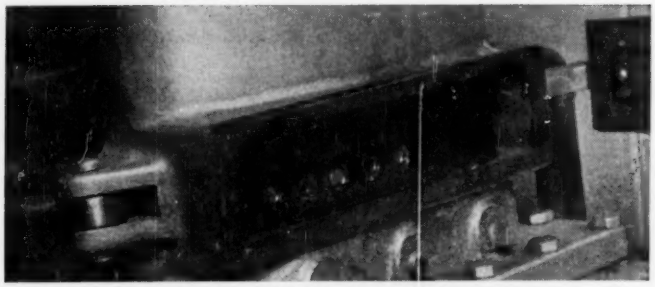

Figure 1. View of cups in thermoresistometer.

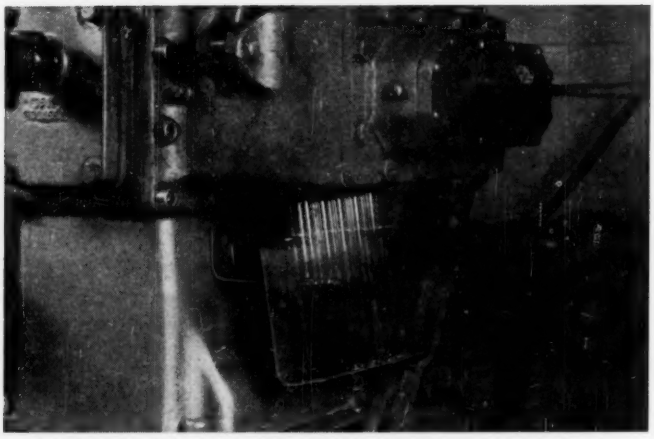

Figure 2. View of cups in tubes under thermoresistometer.

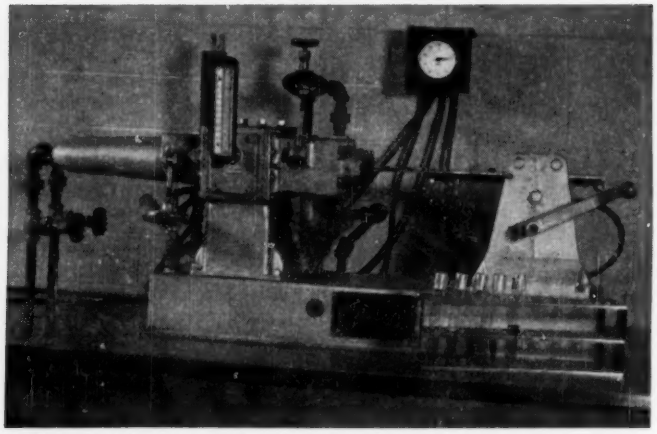

Figure 3. View of cups in tubes in rack under thermoresistometer.

Irradiation. *Cl. botulinum* type B spores were suspended in sterile pork-pea infusion (pH 6.4) to yield approximately 1,000,000 spores per ml. Pork-pea infusion was inoculated with spores of Canco PA-3679 to give an initial count of approximately 8,000,000 spores per ml. Five ml. amounts were added to the polystyrene vials. All vials were frozen and were retained in that state during irradiation and up to the time of culturing.

Samples were radiated at the High Level Gamma Facility, Argonne National Laboratory, where fission products in spent fuel elements provide the source. Vials containing spore suspensions were sealed in 307 x 409 cans for radiation. The radiation dose was determined by ANL personnel from replicate vials of dosimeter solution (0.001 N ferrous sulfate — 0.001 N sodium chloride — 0.8 N sulfuric acid) radiated in 307 x 409 cans in the same position under the same conditions. Dose calculations were based on a molecular yield, $g_{Fe^{+++}} = 15.6$ molecules/100 ev. *(4)*.

Media. The 11 media, as shown in Table 1, were prepared according to the instructions submitted by various individuals. Media 3, 5, 6, and 11 were prepared from infusions made from fresh meat. Pea infusion used in No. 6 was prepared from frozen peas. A sufficient quantity of each infusion was prepared initially for several runs and held at 0°F. Media 1, 2, 7, and 8 were obtained in the dehydrated form, while individual dehydrated ingredients were used for numbers 4, 9, and 10.

The media themselves were prepared in one-liter quantities and then frozen without sterilization. A medium was sterilized only at the time of use and was sterilized according to the instructions (Table 1.) After the initial use, the medium remaining in the container was refrozen and was resterilized for 15 minutes at 250° F. This was done at the time of use for the work under Phase I.

In Phase II, three batches of pork, beef, and pea infusions were made at separate times and held frozen. This was to observe, if possible, whether any significant differences in recoverable counts could be detected from media prepared from different batches of the raw products (meat, peas).

Culturing. Veillon tubes (12 mm. diameter and 20-22 cm. long) made from Pyrex glass tubing were used for all counts. Experiments were performed in randomized blocks of 9, 10, or 11 tubes, depending upon the number of media involved in any one test. Each medium was thus represented once in each block.

The inoculum (1 ml.) was added from a single pipette to the coded tubes (containing 5 or 10% Seitz filtered $NaHCO_3$ where specified), representing a block. Each medium was poured into the seeded tubes in random order, and the entire procedure was repeated 10 times to produce 10 replicate inoculated tubes of each medium. In experiments involving dual incubation temperatures, half of the blocks, 9 to 11 tubes, were selected at random for incubation at 86° F. and the remaining tubes were incubated at 98° F.

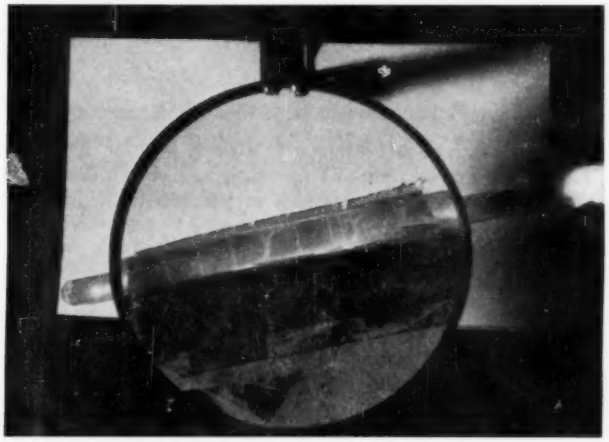

Figure 4. View of Veillon tube on counting chamber.

One extra tube was prepared containing only $NaHCO_3$ for each medium, serving as a control. After the addition of the agar (108°-110° F.) the tubes were stratified either by a vaseline-paraffin mixture or by a specified agar, contingent upon the recommended procedure.

Counting. Incubated tubes were examined over a period up to 6 days. The colonies were counted by placing the Veillon tube across the lighted opening of a modified Quebec colony counter (Figure 4). A metal plate cut out in the shape of a Veillon tube was attached to the Quebec counter to provide support for the tube. Light is thus concentrated only on the counting area of the tube. We are indebted to the National Canners Association for this idea.

RESULTS AND DISCUSSION

General observations. Results of this work are shown in Tables 2-7. Individual tube counts have not been shown because of the volume of tubes (1130) involved. In lieu of individual tube counts, the total colonies recorded for each block of 5 tubes have been shown. Individual tube counts were used in the statistical determinations.

As general observations, Media 2, 8, 10, and 11 have a tendency with some strains to produce gas during the latter part of the incubation period; Media 4, 8, and 10 generally appear to result in larger colony formation in contrast to the smaller colonies developing in Media 3, 5, and 6; Media 1, 7, and 9 have a tendency to cloud or become turbid early in the incubation period due to low agar content.

As shown in Table 2, Media 1, 2, 7, and 9 gave negligible counts for severely heated spores of PA-3679; consequently, these media were not used in Experiment 3.

In general, the near maximum count was obtained by the end of the 72-hour incubation period. Control tubes gave negative results throughout the entire program.

In Table 6 a difference between counts for experiments 14 and 16 is to be observed. The suspensions were irradiated in separate vials in a single 307 × 409 can, with one vial located in the lower half of the can and the other vial in the upper portion.

Statistical Observations

Phase I

1. Total survivor counts for severely heated spores appear in Table 2.

   Four-factor analysis of variance (medium, location in thermoresistometer, number of sterilizations of medium, and repetitions of the experiment) of logarithmic transformations of the 180 individual tube counts led to the following conclusions:

   a. Significant differences exist in recoveries of spores in the various media (.01 significance level). Media 3 and 6 yielded highest counts (Table 8).
   b. In these experiments the 5 slots of the thermoresistometer appeared to be essentially equivalent.
   c. Resterilization of the media did not affect the counts to a significant degree.
   d. The differences among the media were not constant over the 3 repetitions of the experiment (.01 significance level).

2. Total survivor counts for mildly heated spores appear in Table 3.

Four-factor analysis of variance (medium, blocks, number of sterilizations of medium, and repetitions of the experiment) of logarithmic transformations of the 140 individual tube counts led to the following conclusions:

a. Significant differences exist in the recoveries of spores in the various media (.01 significance level). Media 4 and 6 yielded highest counts (Table 8).

b. There was no significant difference among replicate blocks.

c. Resterilization of media did not affect counts to a detectable degree.

d. In this case, all results of the two experiments were entirely consistent.

Phase II

1. Total counts of severely heated PA-3679 in three media, each prepared from 3 infusions, with the entire experiment performed 3 times, are presented in Table 4.

Four-factor analysis of variance (medium, infusion, replicate blocks and repetitions of the experiment) of logarithmic transformations of the 135 individual tube counts led to the following conclusions:

a. The relative performance of the 3 media varied, depending upon the particular lot of raw material used (.05 significance level).

b. Although average counts varied due to different processing conditions on the 3 repetitions of the experiment, relative performance of media and infusions was consistent.

2. Total counts of 2 strains of PA-3679 in 3 media, each prepared from three infusions, are presented in Table 5.

Partial five-factor analysis of variance (strain, medium, infusion, replicate blocks, and experimental run) of logarithmic transformations of 180 individual tube counts led to the following conclusions:

a. Although differing in resistance, the 2 strains responded similarly to the 3 media and to individual infusions.

b. As before, the relative performance of the 3 medium formulations varied with the individual infusion (.01 significance level), but in this case Medium 6 yielded the highest counts (Table 9).

c. In spite of variations due to infusions, there was a consistent difference in survival counts in the 3 media (.05 significance level).

d. There was no significant difference among replicate blocks.

e. Due to differences in thermal treatment, the 4 runs yielded different average counts (.01 significance level.)

Phase III

1. Total counts of irradiated spores of PA-3679 in 11 media incubated at 2 temperatures are presented in Table 6.

The 190 counts of spores which received 540,000 rads were transformed

to logarithms and subjected to four-factor analysis of variance (medium, incubation temperature, replicate blocks, and repetitions of the experiment).

The 55 counts of spores which received 1,540,000 rads were transformed to logarithms and subjected to two-factor analysis of variance (medium and replicate blocks).

a. In each run there were significant differences in counts among media. At 540,000 rads, Medium 2 yielded highest counts. At 1,540,000 rads, there is less data and consequently less discrimination, the highest counts were among Media 10, 8, 5, 2, 7, and 6 (Table 10).
b. The 98° F. counts were significantly higher (.01 significance level) than those at 86° F.
c. In no case were differences noted between replicate blocks.
d. Although counts were higher throughout experiment 14, the relative performance of the various media at both incubation levels was consistent over the two experiments.

2. Total counts of two strains of irradiated *Cl. botulinum* type B, cultured in 11 media and incubated at two temperatures, are presented in Table 7.

Four-factor analysis of variance (strain, medium, incubation temperature, replicate blocks) of logarithmic transformations of the 220 individual tube counts led to the following conclusions:

a. The two strains did not react identically to the media (.01 significance level). The highest counts for Strain 13983-B occurred in Media 6 and 10. The highest counts for Strain 32-B occurred in Media 11, 6, 5, and 10 (Table 10).
b. In spite of differences between strains, there were differences in over-all performance of the various media (.01 significance level).
c. The 98° F. incubation temperature gave higher counts than the 86° F. temperature.
d. There was no significant difference between replicate blocks.

SUMMARY

A comparison of eleven media for the recovery of anaerobic spores indicates that very different counts are obtained, depending upon the medium used. The survival of severely heated spores of PA-3679 was even somewhat influenced by the individual lot of raw material used in the infusion, but the recovery was higher with the three media prepared from infusions than with the dehydrated media. With mildly heated spores, some of the dehydrated media yielded counts comparable to those of media prepared from infusions. The medium yielding consistently higher counts throughout, regardless of strain of PA-3679 used or severity of heat process, was Number 6 (pork-pea agar).

Counts produced by the various media were not significantly affected by one resterilization of the media. Irradiated spores of PA-3679 produced different counts in the eleven media tested. The relative performance of these media, however, was distinctly different with irradiated spores than with heated spores. Medium 2 (Eugonagar with cystine) gave higher counts with

the irradiated spores of PA-3679. Irradiated spores of two strains of *Cl. botulinum* type B on the other hand, yielded higher counts in Medium 6 than in Medium 2. It is of interest to note that recovery counts of both PA-3679 and *Cl. botulinum* type B were higher at 98° F. than at 86° F. for recovery of irradiated spores.

The results of the experiments on both heated and irradiated spores of the test organisms indicated that no one medium appeared to give universally higher recovery counts, as deviations may occur with the strain and type of physical treatment. In order to achieve uniformity in procedures, however, it may prove expedient to select a medium generally resulting in higher counts. Based on the results to date covering a limited number of strains of organisms, the pork-pea agar (Medium 6) is being used by this laboratory for current work.

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IDENTIFICATION OF MICROORGANISMS FROM MAPLE TREE TAPHOLES[a, b]

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(Manuscript received June 18, 1958)

Recent studies *(11, 12)* have established that the growth of microorganisms in maple tree tapholes results in low sap yields. Naghski and Willits *(11)* indicated that bacteria were the most frequent cause of premature stoppage of sap flow, but that yeasts and molds were involved in some instances. Beyond this, there is no information available on the identity of the microflora in the taphole.

Edson *et al. (7)* made an extensive study of the microorganisms in spoiled sap and demonstrated the presence of various types of bacteria, yeasts, and molds. Bacteria of the genera *Bacillus, Micrococcus* and *Pseudomonas* were found in these saps. One species of the bacilli described by these authors, *Bacillus aceris,* was later reclassified as *Achromobacter aceris (3).* The yeasts from the spoiled saps were described only as true yeasts and yeastlike organisms which formed red or grey colonies. Molds of the genera *Penicillium* and *Aspergillus* were identified. Fabian and Buskirk *(8)* reported *Aerobacter aerogenes* as a causative agent of maple sap spoilage, which resulted in ropy sirup.

This work was conducted as a part of an overall program to determine the influence of microorganisms in maple tree tapholes on sap yields. It was necessary to determine which microorganisms were present before their relative importance could be ascertained.

EXPERIMENTAL

All sap samples collected for this investigation were obtained during the 1955 and 1956 seasons of sap production. Maple trees that had never been tapped were selected for the tapping and sampling studies[d]. These were part of a sugar bush on the campus at East Lansing. All samples were collected from the running tapholes or from the collection containers at intervals throughout the 2 seasons, usually during the major sap runs. Samples of sap were taken from normal commercial type tappings and from tappings which were maintained in a closed system *(12)* in an attempt to prevent microbial contamination. Table 1 indicates the number of trees tapped during the 1955 season and the number of samples taken and cultures isolated according to the date of tapping. A total of 1273 cultures was isolated during the 1955 season and an additional 173 were obtained during the 1956 season.

Populations of bacteria and of yeasts and molds were determined in all samples using standard plating and counting techniques. Nutrient agar was used for the enumeration of the bacteria and dextrose agar containing 0.1% yeast extract, acidified with 2 ml. of 5% tartaric acid per 100 ml. of medium, was used for yeast and mold counts.

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[b] This work was conducted under a contract with the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[c] Present address: Wisconsin Malting Co., Manitowoc, Wisconsin.

[d] A more detailed experimental outline of the tapping and sampling methods is presented by Sheneman *et al. (12).*

TABLE 1

Origin of cultures isolated from maple tapholes (1955 season)

Tapping date	Trees tapped	Tapholes sampled	Samples taken during season	Number of cultures [1]
January 10	10	20	200	320
January 25	10	20	200	311
February 10	10	20	180	317
February 25	10	20	140	226
March 10	10	20	100	99
Totals:	50	100	820	1273

[1] Isolations were made from all samples in which microorganisms were found.

All plates were incubated at room temperature for 3 to 5 days. Representative colonies from platings of the highest dilutions were transferred to culture tubes containing appropriate media to preserve the cultures for identification studies. Isolations were made from platings of all samples in which microorganisms were found to be present during the 1955 season.

All cultures isolated were divided into 3 major groups (bacteria, yeasts, and molds) on the basis of colonial appearance and microscopic morphology. The bacteria were examined according to procedures found in *Manual of Methods for Pure Culture Study of Bacteria (4)*, and identified with reference to *Bergey's Manual (3)*. Yeast isolates were further examined and identified according to the system of Lodder and Kreger-Van Rij *(10)*. Methods used were as outlined by these authors except that sporulation tests were run with a vegetable juice (V-8) medium *(9)*, fermentation and assimilation in peptone-yeast extract broth *(9)*, and hyphae production in yeast-morphology agar (Difco). Mold cultures were examined only for morphological characteristics and were identified to genus using Barnett *(1)* and Beneke *(2)* as references.

RESULTS

Population studies of the sap from tapholes demonstrated that both bacteria and yeasts grew in the tapholes. The increase in averages of logarithms of counts per ml. of sap from 30 regular tappings during 1955 is shown in Figure 1. Although the populations of yeasts and molds were not considered separately, it should be noted that molds were rarely found after the first 2 weeks of the season. Therefore, these data did not indicate appreciable mold development in tapholes. However, other data, to be discussed elsewhere, indicate that molds may grow within the tappings and not appear to any extent in the sap.

Bacteria from maple tapholes. The 469 cultures of bacteria isolated during the 1955 season were distributed among 4 major groups (Table 2). As might be expected,

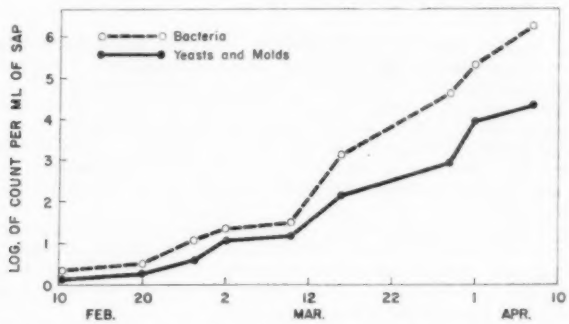

Figure 1. Development of microorganisms in maple tree tapholes as indicated by populations occurring in the sap.

TABLE 2

Bacteria from maple tapholes (1955 season)

Group, genera and species	Number of cultures	Percent of group
I. Gram negative rods (314;67%) [1]		
Pseudomonas		
Ps. geniculata	167	53.2
Ps. putrefaciens	27	8.6
Ps. mephitica	8	2.5
Ps. striata	9	2.9
Miscellaneous	12	3.8
Flavobacterium		
Fl. solare	36	11.5
Miscellaneous	10	3.1
Achromobacter		
A. superficiale	9	2.9
Miscellaneous	27	8.6
Unclassified	9	2.9
II. Gram positive cocci (78;16.6%)		
Micrococcus		
M. varians	18	23.0
M. luteus	15	19.2
M. conglomeratus	9	11.5
M. candidatus	7	9.1
Miscellaneous	24	30.8
Sarcina	5	6.4
III. Gram positive, non-spore forming rods [2] (52;11.1%)		
IV. Gram positive, spore forming rods (25; 5.3%)		
Bacillus		
B. cereus	12	48.0
B. circulans	10	40.0
Miscellaneous	3	12.0
Total: (469; 100%)		

[1] Number of cultures in group and percent of total number.
[2] This was a very heterogeneous group of cultures.

the largest group was comprised of the psychrophilic, gram negative, rod-shaped bacteria of the genera *Pseudomonas, Flavobacterium,* and *Achromobacter. Pseudomonas geniculata* accounted for more than 50% of the cultures in this group, and for about 37% of all the bacteria isolated. The second largest group was comprised of micrococci with at least 4 species of *Micrococcus* and one species of *Sarcina* represented. A fairly large group of gram positive, non-spore forming rods was present. These cultures were not identified since they were very heterogeneous with respect to physiological characteristics and several species were probably represented. Finally, a few cultures of aerobic spore formers were found.

One-hundred and nineteen cultures of bacteria were isolated during the 1956 maple season. Generic classification of these cultures showed 101 (85%) of them to be species of *Pseudomonas;* 5 (4.2%) were *Chromobacterium;* 3 (2.5%) were *Flavobacterium;* 1 (0.8%) *Bacillus;* and 9 cultures (7.6%) were unclassified. The only major difference from the 1955 flora was the absence of *Micrococcus.*

Yeasts from maple tapholes: Nine species representing 5 genera of yeasts were identified among 412 cultures of yeasts isolated during the 1955 season (Table 3). All cultures were found to be non-fermentative, anascosporogenous types. The most fre-

TABLE 3

Yeasts from maple tapholes (1955 season)

Species	Number of cultures	Percent of total
Trichosporon pullulans[1]	84	20.4
Rhodotorula glutinis	75	18.2
Candida sp.	48	11.6
Torulopsis aeria	42	10.2
Cryptococcus laurentii	36	8.7
Cryptococcus diffluens	32	7.8
Cryptococcus albidus	32	7.8
Candida curvata	23	5.6
Torulopsis candida	8	1.9
Unclassified	32	7.8
Totals:	412	100.0

[1] Tentative classification.

quently isolated yeast was one which was tentatively identified as *Trichosporon pullulans*[*]. *Rhodotorula glutinis* was a close second in frequency of isolation. An unidentified species of *Candida* and *Torulopsis aeria* were next in order, followed by 3 species of *Cryptococcus* and *Torulopsis candida*. A fairly large non-uniform group of yeasts remained unclassified.

Fifty-four yeast cultures were isolated during the 1956 studies and were found to be of the same general types. However, the greatest proportion of this collection belonged to the genus *Candida*.

Molds from maple tapholes. The number of molds (370) isolated in 1955 was much out of proportion to their relative numbers in the sap. Practically all of these cultures were obtained early in the season when the total populations of organisms were quite low. The 9 genera found to be represented among these cultures are given in Table 4. *Penicillium* and *Phoma* were the most frequently isolated genera of those identified. However, the majority of the cultures, comprising about 30% of the total, were not identified because of their failure to produce typical fruiting structures on laboratory media. Many of these are probably members of the class *Basidiomycetes*.

There were 22 additional cultures isolated in 1955 which were classed as streptomycetes.

[*] These cultures were identical with the description of this species presented by Lodder and Kreger-Van Rij *(10)* except for the development of a dark (almost black) appearance in old (2-3 weeks) cultures grown on vegetable juice sporulation medium *(9)* or on yeast morphology agar (Difco).

TABLE 4

Molds from maple tapholes (1955 season)

Genus	Number of cultures	Percent of total
Penicillium	83	22.4
Phoma	60	16.2
Hormodendrum	36	9.7
Fusarium	26	7.0
Alternaria	16	4.4
Cephalosporium	13	3.5
Rhizoctonia	11	3.0
Aspergillus	10	2.7
Coniothyrium	6	1.6
Unidentified	109	29.5
Totals:	370	100.0

DISCUSSION

The fact that bacterial populations in sap from maple tapholes are usually greater than the yeasts and molds does not necessarily mean that the former are more important in the stoppage of sap flow. Other experiments in this project have demonstrated that representatives of all 3 groups may result in sap flow stoppage *(12)*. Most yeasts do not develop the high population levels that occur with bacteria. Also, the yeasts may not be as easily washed out of the taphole as the bacteria. It has been demonstrated that molds will grow quite well in the tapholes and result in stoppage of sap flow without any appreciable number appearing in the sap *(12)*. Therefore, it is necessary to consider all organisms capable of growing under these conditions as possible causes of a loss in sap yield.

The microorganisms found in the sap as it comes from the spile are of the same general types known to be responsible for spoilage of sap *(7)*. The *Pseudomonas, Achromobacter,* and *Flavobacterium* groups are common psychrophilic types which are found in water and soil; and, thus, would be expected to develop in conditions such as those encountered in maple tappings. The micrococci and aerobic spore formers might also be anticipated in such an environment.

Trichosporon pullulans has been found in the exudates of birch and chestnut trees, and *Rhodotorula glutinis* has been isolated from air, wood pulp and soil *(10)*. The presence of *Torulopsis aeria, Cryptococcus laurentii, Cyrptococcus albidus* and species of *Candida* would not be considered unusual in this environment. Although the presence of *Cryptococcus diffluens* and of *Candida curvata* was somewhat of a surprise to us, these species have been reported by di Menna *(5, 6)* to be inhabitants of soils in New Zealand. In fact, all of the species identified in the present study except *Torulopsis candida* were found in these soils. It is of particular interest that not a single culture of a fermentative or an ascosporogenous yeast was isolated. Wickerham and Burton *(13)* found a number of fermentative species of 3 related ascosporogenous genera associated with trees, and with insects dependent on both the trees and the yeasts. However, some of these were related only to conifers and slime fluxes of deciduous trees.

SUMMARY

Both bacteria and yeast populations were shown to develop to fairly high levels in maple tree tapholes during the sap season. This was demonstrated by their presence in the sap as it flowed from the tree. Conversely, low mold counts were observed in the sap early in the season, but did not develop to high numbers. This does not prove, however, that the molds did not grow in the tapholes.

Over two-thirds of 588 bacterial cultures isolated were gram negative rods of the genera *Pseudomonas, Achromobacter,* and *Flavobacterium. Pseudomonas geniculata* was the most frequently encountered species. Also present were representatives of the genera *Micrococcus, Bacillus, Sarcina,* and *Chromobacterium,* and a heterogeneous group of non-spore forming, gram positive, rod-shaped bacteria.

Four-hundred and forty-six yeast cultures were isolated during the two

seasons; and, without exception, were all anascosporogenous, non-fermentative types. Of 112 cultures studied in detail, *Trichosporon pullulans* (tentative classification) and *Rhodotorula glutinis* were the most frequently isolated. The remaining cultures were about equally distributed among two species of *Candida*, three of *Cryptococcus*, two of *Torulopsis*, and a miscellaneous group of unidentified yeasts.

A group of 370 mold cultures were studied and 9 different genera recognized among them. However, the largest group of mold cultures (approximately 30 percent) were not identified due to their failure to produce typical fruiting structures on laboratory media.

Acknowledgments

The authors are appreciative of the assistance of Professor P. W. Robbins and Mr. J. E. Douglass, Department of Forestry, Michigan State University, in the tapping and sampling of the maple trees. Dr. E. S. Beneke, Department of Botany and Plant Pathology, Michigan State University, was of much help in the classification of mold cultures.

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CORRELATION BETWEEN MICROBIAL POPULATIONS AND SAP YIELDS FROM MAPLE TREES [a, b, c]

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An early decline and/or stoppage of sap flow from maple tree tapholes frequently occurs during the sap season. This has been commonly attributed to the drying effect of the air or wind on the surfaces of the taphole *(1, 2, 4)*. However, limited studies by Naghski and Willits *(5)* indicated that premature stoppage of sap flow was closely related to an increase in numbers of organisms within the taphole and not to any physical effect. They also proposed that high populations of microorganisms in the taphole would have an adverse effect on the quality of finished syrup.

Since the latter study was on too small a scale to bear statistical analysis, the first experiments in this work were designed to test their findings statistically. The second phase of this work was concerned with the isolation and identification of the various microorganisms occurring in the sap, and is reported elsewhere *(6)*. The next step was to determine what specific microorganisms found in the sap would result in a decline or stoppage of sap flow. Finally, methods of controlling or preventing microbial infection of the taphole were considered in an attempt to find a practical means of controlling microbial activity; and, thus, increase the quality and quantity of sap produced.

EXPERIMENTAL

The experimental work in this investigation was conducted during a three-year period including the 1955, 1956 and 1957 sap production seasons. A maple sugar bush located on the campus at East Lansing, containing previously untapped maple trees, was used for the tapping and sampling studies. Two techniques of tapping were employed. The commercial or regular technique involved using commercial type spiles in 7/16-inch tapholes. The "aseptic" technique was the same as described by Naghski and Willits *(5)* except that sterile 5-gallon cans were used as collection containers instead of the glass carboys. Although all precautions possible were used to exclude microbial contamination, not all of the "aseptic" type tappings were originally in a state of microbial asepsis and none remained in this condition until the very end of the sap seasons. Therefore, "aseptic" as used in this study, refers to this specific tapping procedure and not to the usual definition of this term.

Samples of sap were taken at intervals throughout the 3 seasons, usually during the major sap runs of each season. All samples were taken with sterile equipment, either directly from the running tapholes or from the sap collection containers. The sap produced by the various tappings was measured by weighing to the nearest tenth of a pound each day on which a sap run occurred.

Populations of bacteria, yeasts and molds were determined in all samples using standard plating and counting techniques. Nutrient agar was used for the enumeration of the

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[b] Presented at the 18th Annual Meeting of IFT, Chicago, May 27, 1958.

[c] This work was conducted under a contract with the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

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[e] Present address: Southeast Forest Experiment Station, Ashville, North Carolina.

bacteria; and dextrose agar containing 0.1% yeast extract and acidified with 2 ml. of 5% tartaric acid per 100 ml. was used for yeast and mold counts. All plates were incubated at room temperature for 3 to 5 days.

RESULTS

Comparison of "Aseptic" and regular tappings. To investigate the relationship existing between premature decline in sap production and microbial activity within the tapholes, "aseptic" and regular tappings on the same trees were compared with respect to microbial populations in the sap and yields of sap. The effect of various dates of tapping on microbial activity in the tappings and on sap yields was also studied during the 1955 season.

Ten trees were tapped on each of 5 dates spaced at approximately 15-day intervals beginning on January 10, 1955. The dates were chosen so that the first 3 dates would fall before the normal tapping period for the East Lansing area, the fourth date during this period, and the last date after the normal tapping time. Each tree was tapped with an "aseptic" and a regular tapping 6 inches apart on the same quadrant of the tree according to compass position. Microbial populations in the sap and sap yields were determined throughout the season.

The "aseptic" tapping procedure was quite successful in delaying the appearance of high populations of microorganisms in tapholes bored early in the season. Thus, the average microbial populations in the sap from the regular tappings made January 10, January 25 and February 10 had attained high levels prior to any appreciable numbers appearing in the sap from the "aseptic" tappings (Fig. 1). However, the difference between the 2

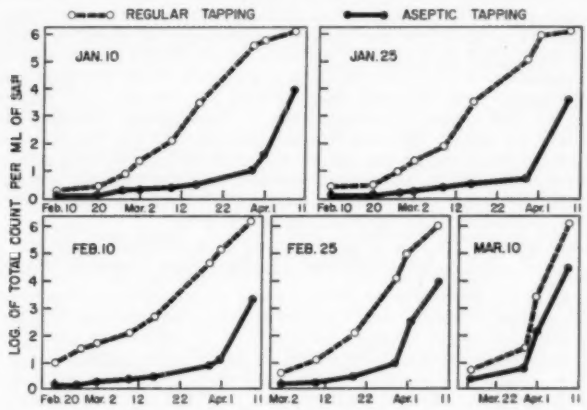

Figure 1. Populations of microorganisms in sap from regular and "aseptic" tappings.

types of tappings was much less in the February 25 tappings; and was not significant in the March 10 tappings. This demonstrates that the so-called "aseptic" tappings were not sterile; and that the relatively warm weather occurring during the last part of the sap season encouraged rapid growth of microorganisms in both types of tappings.

High populations of microorganisms early in the season in the sap from tapholes were accompanied by low average yields. This effect is illustrated by the percent increase in yield resulting from "aseptic" tapping procedures (Fig. 2). These percentages were calculated as follows:

$$\% = \frac{\text{lbs sap from "aseptic"} - \text{lbs sap from regular}}{\text{lbs sap from regular}} \times 100$$

The increased yield obtained by "aseptic" tapping is quite evident for the first 3 tapping dates; but was insignificant for the last two. Analysis of variance of the yield data demon-

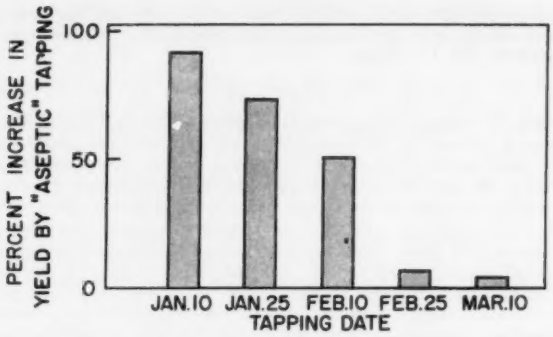

Figure 2. Increased sap yield resulting from "aseptic" tapping.

strated a highly significant difference (1% level) between the 2 tapping techniques for the January 10 tapping date; and significant differences (5% level) for the January 25 and February 10 tappings.

To obtain a statistical evaluation of the correlation between increased microbial activity within the taphole and the decrease in yield resulting from this activity, it was first necessary to establish a microbial population level in the sap considered to represent a condition of incipient decline in yield from the taphole. Examination of microbial population data indicated that 10^4 organisms per ml. of sap could be considered to represent a high level of microbial activity in the taphole which would eventually result in stoppage of sap flow. A correlation analysis was computed using all data for "aseptic" and regular tappings from the 1955 and 1956 seasons. The percent increased yield produced by the "aseptic" (A) as compared with the regular (R) tapping was correlated with the days difference between dates of attainment of 10^4 organisms per ml. of sap in the "aseptic" and regular tappings. Figure 3 shows the predicting line and its equation, together with the standard error of estimate of this predicting line. A correlation coefficient of 0.66 was calculated. This correlation was determined to be significant at the 1% level.

Inoculation Studies: To further test the effect of microorganisms on sap yields from maple trees and to ascertain what particular organisms were responsible for the stoppage

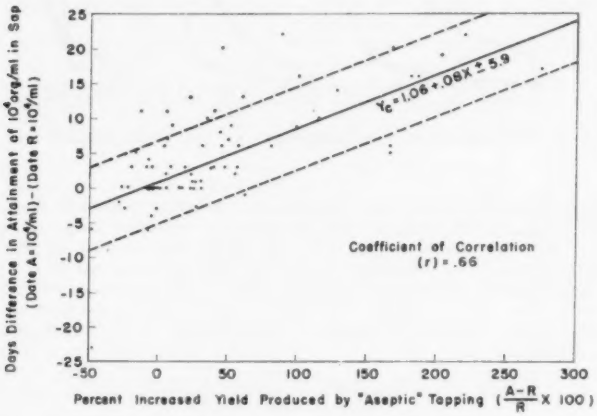

Figure 3. Correlation of differences in microbial infection with differences in sap yield from "aseptic" and regular tappings.

of sap flow, an inoculated tapping was included in the second season's work. Therefore, each tree had 3 tapholes spaced 6 inches apart: one regular, one "aseptic," and the third tapped by the "aseptic" procedure but inoculated with 2 ml. of a suspension of one or more microorganisms. The cultures used were those found to be most prevalent in sap from tapholes *(6)*. Four trees were inoculated with *Pseudomonas geniculata*, and 2 trees with each of the following: *Pseudomonas putrefaciens, Flavobacterium solare, Achromobacter sp., Micrococcus varians, Candida sp., Rhodotorula glutinis, Torulopsis aeria, Cryptococcus laurentii,* a culture of a large group similar to *Trichosporon pullulans, Penicillium sp.*, a mixture of the five yeast cultures, a mixture of the five bacteria cultures, and a mixture of all bacteria and yeast cultures. Of the total 30 trees tapped, 15 were tapped February 7, and 15 on February 29.

Microbiological studies of sap from these tappings were made throughout the season. In general, the sap from tapholes inoculated with bacteria had initial populations of about 10^4 per ml. and final populations of about 10^7; and that from tapholes inoculated with yeasts had initial levels of about 10^3 and final levels of about 5×10^5. There was a fairly constant increase in the number of these organisms throughout the season. The sap from the 2 tapholes inoculated with mold did not develop high numbers of molds. However, reamings from these tapholes made at the close of the season revealed that they were heavily infested with mold hyphae.

No high microbial numbers were noted in the sap from either the regular or "aseptic" tappings until very late in the season. This probably resulted from the uniformly cold weather during February and most of March, 1956.

Effect of taphole inoculations on sap yield is shown in Table 1. The inoculated tappings of February 7 yielded only about one-third of the total amount of sap produced by the "aseptic" tappings, whereas the inoculated tappings of February 29 produced about one-half of the amount of sap from the "aseptic" tapholes. Analysis of variance calculations with these data showed a highly significant difference between yields of regular and inoculated tappings and between yields of "aseptic" and inoculated tappings. There was about a 22% greater yield from the "aseptic" than from the regular tapholes, but this did not prove to be significant. This might be expected due to the relatively low numbers of microorganisms observed in the regular tappings of this season.

TABLE 1

Sap yields from "aseptic", regular, and inoculated tappings. (1956 season)

Date of tapping	Average yield in pounds of sap		
	"Aseptic"	Regular	Inoculated
Feb. 7	152.4	126.7	57.3
Feb. 29	149.8	121.1	77.9
Average	151.1	123.9	67.6 **

** Significant at the 1% level.

Of particular interest was the fact that all microorganisms tested reduced sap flow. Every inoculated tapping had either completely stopped producing sap or had appreciably diminished in flow at least 2 weeks before the uninoculated tappings. This appears to be a very non-specific effect insofar as the type of microorganism is concerned.

A correlation coefficient was obtained from data similar to those shown in Figure 1 for the "aseptic" and inoculated tappings. The equation of the predicting line was found to be $Yc = 28.92 + .03 \times \pm 6.7$. The coefficient of correlation (r) was 0.58, which is significant at the 1% level.

Treatments of tapholes to prevent microbial growth or contamination. The "aseptic" tapping technique would be impractical under commercial conditions. Therefore, studies were conducted with microbial inhibitors and sanitizers added to the tapholes. Also, an attempt was made to reduce the amount of microbial contamination of the taphole by the use of plastic tubing running from the spiles to the collection containers.

Preliminary studies using aureomycin and hypochlorite solutions as rinses and inserting sorbic acid pellets in tapholes proved to be unsuccessful in controlling microbial growth in the tapholes. This might have been expected since the sorbic acid is relatively ineffective at the pH of sap, the aureomycin has no effect on the yeasts and molds, and the hypochlorite is destroyed very rapidly in the presence of organic material.

More drastic treatments for controlling the microorganisms were then resorted to. These were: (a) paraformaldehyde (trioxymethylene) in agar pellets, (b) paraformaldehyde in plaster of paris pellets, (c) mercuric iodide in plaster of paris pellets, (d) "Sterilite"[f] powder added directly to the taphole, and (e) "O-Silver" aqueous[g] applied in suspension to cotton stuffed in the tapholes. A group of 14 trees was tapped for each of these treatments with each tree having a treated and an untreated taphole. Both the control and treated tappings in 7 trees of each group were inoculated with 2 ml. of a broth culture of *Ps. geniculata.*

Microbiological studies showed that all treatments, except the "O-Silver" suspension, did an excellent job of preventing growth of microorganisms in the tapholes. The type of control obtained is illustrated by the data for the trees treated with paraformaldehyde-agar pellets in Figure 4. While little or no control was evident with the "O-Silver" treatment (Fig. 4), this may well have been due to the method of treatment.

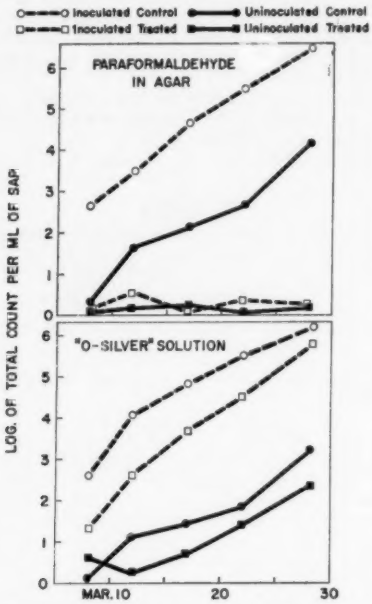

Figure 4. Effect of paraformaldehyde and "O-Silver" on the development of microorganisms in maple tree tapholes.

An additional experiment was run testing the effect of using Tygon plastic tubing to conduct the sap from the spile to the collection container. No inoculations were used in these experiments. Tubing which had been treated with silicone was used on the treated tapping on seven of the trees in this experiment and regular tubing on the other seven. The data indicated some reduction in number of microorganisms in the sap from the tapholes with plastic tubing. However, the control was far from complete and considerable variation was noted among replications.

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[f] The "Sterilite" powder, a preparation of colloidal carbon coated with colloidal silver, was supplied by the Shellmar-Betner Flexible Packaging Division of Continental Can Co., Inc., Mount Vernon, Ohio.

[g] The "O-Silver" aqueous, a germicidal suspension of oligodynamic silver, was supplied by The Chloromine Co., 54 West 16th Street, New York 11, New York.

Yield data were collected for all the treated tappings except for those treated with mercuric iodide. The effect of the two paraformaldehyde treatments and the "Sterilite" powder treatment on yield of sap was quite pronounced (Table 2). An analysis of variance of these data showed that the yields from these three treatments were significantly higher than the corresponding controls. In fact, the average yield from tapholes treated with the paraformaldehyde in agar was approximately four times that from the corresponding controls. The "O-Silver" and plastic tubing treatments did not result in significantly greater yields.

TABLE 2

Effect of various treatments on average yields of maple sap

Treatment [1]	Inoculation	Average yields in pounds	
		Treated	Untreated
Paraformaldehyde in agar	Uninoculated [2]	319.8	105.1
	Inoculated	349.4	64.0
	Average	334.6	84.5 **
Paraformaldehyde in plaster	Uninoculated [2]	271.4	191.0
	Inoculated	305.6	74.8
	Average	288.5	132.9 *
Sterilite powder	Uninoculated [2]	243.3	202.7
	Inoculated	260.9	116.1
	Average	252.1	159.4 **
O-Silver solution	Uninoculated [2]	138.7	172.4
	Inoculated	99.4	87.4
	Average	118.6	129.9
Plastic tubing	Silicone coated [3]	191.1	154.0
	Uncoated	257.7	190.9
	Average	224.4	172.5

[1] Fourteen trees were tapped per treatment with a treated and a control tapping in each tree.

[2] Both tapholes were inoculated on 7 trees and uninoculated on the other seven.

[3] The plastic tubing used on the treated tappings was silicone coated on 7 trees and not coated on the other seven.

** Significant at the 1% level.

* Significant at the 5% level.

DISCUSSION

Growth of microorganisms (bacteria, yeasts, or molds) in maple tree tapholes results in a decline in flow rate and, frequently, complete stoppage of sap flow. These data confirm the preliminary observations of Naghski and Willits *(5)*, and refute the popular belief that the physical drying by air and wind causes premature stoppage of maple sap flows. The extent of this effect on sap yield will vary greatly from tree to tree and with the season. The tree variation is probably the result of differences in initial contamination and the rate of infection of the tissues around the taphole. Differences in average temperatures of sap seasons would undoubtedly influence the rate of development of microorganisms within the taphole. Such a difference was indicated in this study by comparison of the data for the 1955 season with that of 1956. The season was quite cold in 1956 compared to 1955, and microbial development in the regular tapholes of 1956 did not occur nearly as early as in 1955. In correlation with this, the average yield differences between the two types of tappings made early in February was significant in 1955 but not in 1956. However, when the initital populations of microorganisms were high, as a result of inoculation, yields were greatly reduced even in a relatively cold

season. The amount of precipitation and wind occurring during the season may have an influence on the amount of natural contamination of tapholes; and, thus, indirectly influence yields. Still another variable, is the time of exposure of the taphole to natural contamination and to growth of microorganisms. Yields from trees tapped early in the season are much more likely to be affected by microbial growth than trees tapped at later dates.

The actual mechanism by which microorganisms stop the flow of sap from maple tree tapholes is not known. However, the fact that so many unrelated types and species of microorganisms will result in stoppage indicates that the effect may be a purely physical one. Histological studies of the tissues around infected tapholes conducted in conjunction with this work showed bacteria and yeast cells, and fungal hyphae in vessels and other types of cells *(3)*. Gummy substances containing numerous bacteria were frequently found plugging the open lumens and vessel elements of the tissue.

The control of microbial development in tapholes is a difficult problem. The tapholes are quite subject to initial contamination from the bark and surface tissue and to subsequent contamination through the spile and around the edges of the spile. The most promising method of control is the use of some good antimicrobial agent in a form that could be inserted into the taphole when it is bored. This would have to be incorporated in a pellet with a slow disintegration rate so there would be a residual in the taphole throughout the season. Also, this agent should either be volatilized or destroyed during the concentration of the sap to syrup. Paraformaldehyde incorporated into a pellet does a fine job of microbial control, but there is insufficient evidence available at this time with respect to its elimination from the sap during concentration. This is undergoing further investigation.

SUMMARY

A comparison has been made of microbial populations and sap yields from regular and experimentally inoculated tapholes in maple trees with tappings in which the development of microorganisms was partially or completely controlled by the use of special tapping procedures or chemical additives. This study was conducted over a three-year period, and was of sufficient scope to permit statistical analysis. A high degree of correlation was demonstrated between the occurrence of high populations of bacteria early in the sap season and low yields of sap. Therefore, it is concluded that microorganisms are the primary cause of premature stoppage of sap flow.

Inoculation studies with a number of species of bacteria and yeasts and one mold culture demonstrated that any organism capable of growing in the environment would result in stoppage of sap flow. Therefore, it is indicated that the organisms probably stop sap flow by physical blocking of the vessels. The amount of initial contamination and the average temperature affect the rate of development of the microflora; and, thus, influence the time at which a drying up of the taphole occurs.

Use of a pellet containing paraformaldehyde inserted into the taphole was the most promising of a number of methods tested for controlling microorganisms. However, insufficient data are available on the elimination of the formaldehyde from the sap during concentration to syrup to make any conclusions as to its usefulness at this time.

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FREE AMINO ACIDS IN CITRUS AND OTHER FRUIT AND VEGETABLE JUICES

The differentiation of the so-called "essential" and "nonessential" amino acids has been based largely upon an extended series of investigations with young, adult male subjects by Rose and coworkers *(21* to *24)*. More recent studies on the amino acid requirements of women have been reported by Leverton *et al. (2* to *8)*, Jones *et al. (1)* and Swendseid *et al. (25, 26)*. A sharp distinction has been drawn between the nutritional importance of the "essential" in comparison with the assumed dispensability of the other naturally occurring amino acids. Some of the principal arguments opposed to an over-emphasis of the importance of the so-called "essential" amino acids include: a) unsupplemented rations containing an excess of the "essential" amino acids fail to support optimum growth, reproduction and survival of young, while the addition of "nonessential" amino acids produce marked improvements in the over all performance of experimental animals *(11, 17)*; b) the maintenance of nitrogen balance is not necessarily an adequate and sufficient criterion of the nutritional adequacy of a diet in terms of general health and well being *(13* to *15)*; c) isotope tracer studies indicate that both "essential" and "nonessential" amino acids are synthesized during normal metabolism, the primary differences being quantitative rather than qualitative *(9)*; and d) bacterial syntheses in the alimentary tract *(12, 16)* or the presence of "nonessential" amino acids in the unknown nitrogen fraction of the diet would obscure recognition of the nutritional significance of these compounds. In the latter connection it was interesting to note that the basal diet employed by Rose's group contained 100 ml. daily portions of filtered lemon juice. The total unknown nitrogen in the diets ranged from 0.3 to 0.4 g./day, the equivalent of 10% of the total or about 25% of the "effective" nitrogen *(23)*.

During the course of investigations on the nitrogenous constituents in citrus fruits *(10, 19, 20, 28)* it was observed that the major nitrogenous compounds in filtered citrus juices were limited almost exclusively to those free amino acids which Rose and coworkers have classified as "nonessential". It was also of interest that Leverton's and Swendseid's basal diets also included lemon juice as well as appreciable quantities of other natural products such as orange, apple, grape and tomato juices, peaches, pears, carrots, etc. Therefore, it appeared appropriate to examine a number of fruit and vegetable juices and extracts to determine whether these products contained significant amounts of the so-called "nonessential" amino acids. The free amino acids observed in the juices or aqueous extracts of 4 vegetable and 6 fruit products, estimated qualitatively with two-directional paper chromatography *(18)*, are presented in Table 1. It may be seen that as in citrus juices, the "nonessential" amino acids predominate. Quantitative chromatographic estimations *(19)* of seven free amino acids in various citrus juices are summarized in Table 2. It is clear from a comparison of these data with the reported nutritional requirements for humans, summarized in Table 3, that sufficient amounts of some of the so-called "nonessential" amino acids were probably present in the basal diets and which would prevent the recognition of the nutritional importance of these compounds or of their possible essentiality at levels

TABLE 1

Relative amounts [1] of prominent free amino acids observed on paper chromatograms of various filtered fruit and vegetable juices or extracts

Amino Acid	Vegetable				Fruit					
	Carrot	Beet	Celery	Cabbage	Cherry	Pineapple	Persimmon	Grape (canned)	Apple	Strawberry
Alanine	*	***	*	**	**	**	*	**	**	**
Glutamic acid	**	*	*	*	*	*	*	**	**	*
Aspartic acid	*	*	*	*	*	*	*	*	*	*
Serine	*	**	*	*	*	*	**	*		*
γ-Aminobutyric acid	*	*	*		*	**		*		
Arginine	*						**	**		
Proline	***	*			***		***		***	
Glutamine	***	****	**	***	**		**		*	**
Asparagine	*	**	**	**	**	**			*	**
Pipecolic acid									**	
Histidine	?									
Methionine-valine or tryptophan			?		?	?				

[1] Amounts, indicated by * marks, are relative only to other amino acids in the same sample.

TABLE 2

Seven free amino acids in the filtered juices of California Eureke lemons, California Valencia and Washington Navel oranges

Amino Acid	Eureke lemon [1]		Valencia orange [2]		Navel orange [2]	
	Range	Average	Range	Average	Range	Average
	mg./100 ml.	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*	*mg./100 ml.*
γ-Aminobutyric acid	4 to 20	7	9 to 73	30	21 to 38	29
Alanine	1 to 31	12	13 to 25	17	18	18
Serine	12 to 28	17	4 to 36	15	23 to 30	26
Glutamic acid	6 to 35	21	19 to 71	49	19 to 21	20
Aspartic acid	19 to 60	38	32 to 94	71	41 to 44	42
Proline	27 to 53	40	69 to 250	123	103 to 295	199
Arginine	25 to 106	45	90 to 150	120	73 to 82	77

[1] Fifteen miscellaneous samples of tree-ripe and "sweated" Eureka lemons.
[2] Taken from Rockland and Underwood *(19)*. The average values represent the means of values presented by the latter authors and Wedding and Sinclair *(29)*.

TABLE 3

Summary of the amino acid requirements for the maintenance of nitrogen balance in young adult human subjects

Amino Acid	Range of requirements for individual subjects	
	Men (21)	Women
	mg./day	*mg./day*
L-Tryptophan	150 to 250	82 to 157 *(8)*
L-Threonine	300 to 500	73 to 305 *(3, 6)*
L-Lysine	400 to 800	630 [1] *(1)*
L-Valine	400 to 800	275 to 650 *(4, 5)*
L-Leucine	500 to 1100	170 to 710 *(2)*
L-Isoleucine	650 to 700	250 to 450 *(25)*
L-Phenylalanine	800 to 1100	120 to 220, with 900 mg. tyrosine *(7)*
L-Methionine	800 to 1100	150 to 230, with 500 mg. cystine *(1)* 180 to 550, with 200 mg. cystine *(26)*

[1] Adequate for all subjects (mature women).

perhaps somewhat below that observed for the currently accepted "essential" amino acids.

Tuttle *et al. (27)* recently stated that "While small supplements have perhaps lowered the need for essential amino acids in animals we have some highly suggestive evidence that this is not true when larger amounts of non-essential nitrogen are added to the diets of humans". The same authors suggest that elderly men have a higher requirement for one or more of the essential amino acids than younger individuals or that the supplementary source of nitrogen may not be as readily available to them for synthesis of non-essentials as is the case in young adults.

In no case has optimal performance of human subjects been demonstrated on diets which are devoid of the so-called "nonessential" amino acids. Our present knowledge does not preclude the probable practical significance in

human nutrition of some of the individual amino acids currently assumed to be "nonessential".

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THE LOCUS OF AROMA IN THE MUSHROOM
(Agaricus campestris L.)

Prior to a chemical examination of the flavor components of the common field mushroom *(Agaricus campestris L.)*, it was deemed desirable to conduct a sensory examination of mushrooms in order to determine whether the aroma typically associated with these fungi was more prevalent in one or more of the particulate parts of the fruiting body. In the event certain tissues were richer in aroma, this knowledge would facilitate the concentration of starting material for subsequent chemical investigation.

The mushrooms used in this study were grown commercially in the Davis, California area. They were harvested just past the button stage of maturity, as indicated by observing that the velum (veil) had begun to rupture near its juncture with the pileus (cap). Immediately after harvesting, the mushrooms were brought to the laboratory where they were chilled, thoroughly rinsed in ice-cold water, drained and prepared for testing. A period of approximately 12 hours elapsed between the time of harvest and examination.

For the sensory examination, the mushrooms were divided into five morphological entities: the intact pileus (cap), the intact stipe (stem), the gills, the pileus cuticle, and the stipe cuticle. These parts were chopped finely, and 2.0 g. each of the fresh tissues described above were placed in eight-ounce tumblers which had been previously coated with an odorless, inert, black enamel to exclude light, and thus preclude the effects of visual judgment upon the odor values. The tumblers were covered with aluminum caps, and the aroma examinations conducted immediately. The examinations were made by the method of paired comparisons. Two of each of the five pairs of the combinations, as indicated in Table 1, were evaluated at each of two sessions by each examiner. A total of 510 judgments was obtained from members of the laboratory staff using as the criterion for the comparison, "Which sample of the two has the most pronounced mushroom aroma?"

A summary of the results as presented in Table 1 indicates that no statistically significant *(1)* aroma difference was detected between the pileus and the stipe and the pileus and the pileus cuticle. There was, however, a marked aroma difference between the stipe and the gills, the stipe and the stipe cuticle, and the pileus and the gills.

Another experiment was conducted in which fresh intact pileus and stipe tissues were prepared as described above, and 2.0 g. portions were weighed into

TABLE 1

Comparison of mushroom aroma intensity in various parts of the raw mushroom (Agaricus campestris L.)
Number of judgments assigning more intense aroma

	Pileus vs. Stipe		Stipe vs. Gills		Pileus vs. Pileus cuticle		Stipe vs. Stipe cuticle		Pileus vs. Gills	
Number of selections	58.5 [1]	43.5 [1]	72 [2]	30	47	55	38	64 [3]	84 [2]	18

[1] The number 0.5 indicates that in this pair one examiner could make no distinction between the two samples, hence his choice was divided between the two.
[2] A probability factor of 0.001 (1).
[3] A probability factor of 0.01 (1).

250 ml. beakers. The beakers were covered with aluminum caps and heated for ten minutes on a steam bath. We observed that the warming markedly changed the character and intensity of the mushroom aroma. A typical "cooked" mushroom odor was evident. The samples were cooled to room temperature, and the relative intensity of mushroom aroma between the two samples was evaluated by the paired comparison method as described above. In this experiment 192 comparisons were made by 24 examiners, each of whom was asked to select the sample with the more intense mushroom aroma in a series of 8 comparisons. A summary of the results is presented in Table 2. From these data it can be seen that no statistically significant (1)

TABLE 2

Comparison of cooked mushroom aroma intensity in pileus and stipe of steam mushrooms (Agaricus campestris L.)

	Pileus	Stipe
No. of judgments assigning more intense aroma	94.5 [1]	97.5 [1]

[1] See footnote 1 for Table 1.

difference was detected between the aroma intensities of the pileus and the stipe in the cooked mushrooms examined.

Thus it may be concluded that the major residences of aroma in the common field mushroom *(Agaricus campestris L.)* are the pileus trama (the central portion of the pileus exclusive of the cuticle) and the context (the central portion of the stipe exclusive of the cuticle). The hymenium and gill trama (gills) and cuticular tissue appear to be far less aromatic on an equal tissue weight basis than the pileus and stipe. There was no statistically significant difference *(1)* between the pileus and stipe as far as aroma intensity is concerned, since no discrimination could be made between the two in either the raw or cooked state.

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